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Characterization in *Drosophila melanogaster* of dPDZ-GEF, a Rap GTPase activator

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Résumé

Le maintien de l'équilibre au sein d'une cellule dépend en partie d'une stricte régulation de plusieurs processus dont l'adhésion, la migration, la prolifération et la survie cellulaires. Un déséquilibre, quoi qu'il puisse être, peut entraîner une cascade d'évènements cellulaires pouvant donner lieu à plusieurs pathologies, incluant le cancer. Par exemple, des mutations activatrices chez certains membres de la voie Ras-MAPK sont étroitement associées à la formation de tumeurs cancéreuses chez 30% des cancers humains. Le laboratoire du Dr. Therrien étudie la fonction de certains membres cette voie chez *Drosophila melanogaster*, soit l'un des organismes modèles les plus fréquemment utilisés dans l'étude des voies de signalisation.

Nous avons identifié des mutations dans les gènes encodant la petite GTPase Rap, ainsi que son activateur PDZ-GEF lors d'un crible génétique réalisé chez la drosophile dans le but d'identifier des partenaires d'une composante clé de la voie Ras-MAPK, soit CNK. Étonnamment, dPDZ-GEF est le seul activateur de Rap ayant été isolé du crible, ce qui suggère que la relation entre Rap et CNK dépend potentiellement de la fonction de dPDZ-GEF. Par suite, le but de mon projet de maîtrise a été d'étudier la fonction de dPDZ-GEF chez la drosophile. Pour ce faire, un des effets phénotypiques des allèles issus du crible, soit un défaut d'espacement des poils sensoriels de la marge antérieure de l'aile adulte, a été principalement caractérisé.

Au cours de cette étude, il a été montré que les allèles de *dPDZ-GEF* perturbent la morphologie et la localisation des cellules appartenant aux organes sensoriels au cours du développement de l'aile. De plus, les résultats obtenus démontrent que dPDZ-GEF est impliquée dans la localisation des sous-unités α et β des intégrines, et potentiellement dans celle des protéines qui forment les jonctions adhérentes. Finalement, une intéraction génétique entre CNK et dPDZ-GEF a été dévoilé dans la marge antérieure de l'aile.

De ces faits, les conclusions suivantes peuvent être apportées: dPDZ-GEF est impliquée dans la distribution de molécules contrôlant l'adhésion cellulaire, et son activité

serait directement ou parallèlement associée à celle de CNK afin de promouvoir la transduction de signaux au cours du développement chez la drosophile.

Mots-clés : PDZ-GEF, Rap, CNK, transduction de signaux, adhesion cellulaire, cadhérine, intégrine, marge de l'aile, organe sensoriel, drosophile

Abstract

Proper regulation of cell adhesion, migration, proliferation and survival is essential for the maintenance of cellular equilibrium. Any upset of that balance, however small it is, can precipitate cells into a chain of events that can lead to numerous pathologies, including cancer. For instance, activating mutations in some members of the Ras-MAPK pathway are involved in the formation of tumours in 30% of all human cancers. Dr. Therrien's laboratory studies the function of members of the Ras-MAPK pathway in *Drosophila melanogaster*, one of the most frequently used model organisms in the study of signal transduction.

Alleles of the genes encoding the small GTPase Rap and its activator PDZ-GEF were isolated from a genetic screen conducted in *Drosophila* in order to identify molecules that can interact with CNK, a key regulator of the Ras-MAPK pathway. Interestingly, dPDZ-GEF was the only known Rap activator for which alleles were isolated, suggesting that the relationship between CNK and Rap may depend on dPDZ-GEF function. In consequence, the aim of my Master's research project was to study the function of dPDZ-GEF by characterizing primarily one of the phenotypic effects of dPDZ-GEF mutations in *Drosophila*, being a spacing defect affecting sensory bristles in the anterior wing margin of adult flies.

During the course of this study, it was demonstrated that *dPDZ-GEF* mutant alleles disturb the morphology and the localization of sensory organ cells during wing margin development. dPDZ-GEF has proven to be involved in the localization of α and β integrin subunits, and possibly, of adherens junction proteins. Moreover, CNK and dPDZ-GEF were shown to genetically interact within the anterior wing margin.

Therefore, the overall conclusions are that dPDZ-GEF is involved in the distribution of adhesion molecules and that it may function with or in parallel to CNK in order to promote signal transduction during developmental processes in *Drosophila*.

Keywords : PDZ-GEF, Rap, CNK, signal transduction, cell adhesion, cadherin, integrin, wing margin, sensory organ, *Drosophila*

Table of contents

List of figures

Abbreviations

AEL	After egg laying
AF-6	Afadin
APC	Adenomatous polyposis coli
APF	After pupal formation
B1-AR	beta 1-adrenergic receptor
BCAR-3	Breast cancer antiestrogen resistance 3
BCR/ABL	Breakpoint cluster region/Abelson protein
C (Cys)	Cystein
C3G	CRK SH3-binding GNRP
CalDAG-GEF	Calcium Diacyl glycerol guanine nucleotide exchange factor
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophosphate
cNMP	Cyclic nucleotide monophosphate binding domain
CNK	Connector enhancer of KSR
CNK^{CT}	Caboxy-terminal CNK
Ct	Cut
DAG	Diacyl glycerol
DEP	Dishevelled/Egl-10/Pleckstrin domain
DER	*Drosophila* epidermal growth factor receptor
Dlg	Discs large
DNA	Deoxyribonucleic acid
DOCK4	Dedicator of cytokinesis 4
dsRNA	Double stranded ribonucleic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMS	Ethyl methanesulphonate
Epac	Exchange protein directly activated by cAMP
EPO	Erythropoietin
ER	Endoplasmic reticulum
ERK	Extracellular regulated MAP kinase
EtOH	Ethanol
F-actin	Filamentous actin
FGF	Fibroblast growth factor
GAP	GTP-activating protein
Gbp	Guanosine monophosphate binding protein
G-CSF	granulocyte-colony stimulating factor

GDP	Guanine nucleotide diphosphate
GEF	Guanine nucleotide exchange factor
GPCR	G protein-coupled receptor
GST	Glutathione-s-transferase
GTP	Guanine nucleotide triphosphate
GTPase	GTP-binding protein
Hh	Hedgehog
HOG	High osmolarity glycerol
HRP	Horseradish peroxidase
HUVEC	Human umbilical vein endothelial cells
HYP/Ave	Hyphen/Aveugle
ICAM	Intercellular adhesion molecule
IgG	Immunoglobulin
IL-3	Interleukin-3
JNK	c-jun N-terminal kinase
Kb	Kilobase
kDa	Kilodaltons
Krev1	K-Ras revertant-1
KSR	Kinase suppressor of Ras
Leu	Leucine
MAGI	Membrane associated guanylate kinase inverted
MAGUIN	Membrane-associated guanylate kinase-interacting protein
MAPK	Mitogen-activated protein kinase
MBP	Maltose-binding protein
mg	Milligram
min	Minutes
mL	Milliliters
mM	Millimolar
NGF	Nerve growth factor
OL	Oligonucleotide
PDGF	Platelet-derived growth factor
PDZ	PSD-95/Dlg/ZO-1
Phe	Phenylalanine
Phg2	Phagocytosis 2
PKA	Protein kinase A
PKC	Protein kinase C
PKG	Protein kinase G
PLC	Phospholipase C

PPXY	Proline-Proline-X-Tyrosine (X is any residue)
R	Roughened
RA	Ras/Rap1 associating
R cells	Receptor cells
RalGDS	Ral guanine nucleotide dissociation stimulator
Rap	Ras-like protein
RAPL	Regulator for cell adhesion and polarization enriched in lymphoid tissues
Ras	Rat sarcoma
RASSF1	Ras association domain family-1
REM	Ras-exchange motif
Repac/GFR/MR-GEF	Related to Epac/M-Ras regulated Rap GEF
RIR	Raf inhibitory region
RNAi	Ribonucleic acid interference
RTK	Receptor tyrosine kinase
RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
Sca	Scabrous
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
sE	Sevenless enhancer
SH2/SH3	Src homology-2/3
Shc	Src homology 2 domain containing
siRNA	Small interfering ribonucleic acid
SOP	Sensory organ precursor
SPA-1	Signal-induced proliferation-associated protein-1
S-SCAM	Synaptic scaffolding molecule
TCR	T-cell receptor
Tor	Torso
TPO	Thrombopoietin
VCAM	Vascular cell adhesion molecule
VE-cadherin	Vascular endothelial-cadherin
VEGF	Vascular endothelial growth factor
VSAV	Valine-Serine-Alanine-Valine
Wg	Wingless
ZO-1	Zonula occuldens

Je dédie ce mémoire à ma mère pour qui l'accomplissment de ma maîtrise a autant de valeur que pour moi

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CHAPTER 1 : INTRODUCTION

Introduction

Throughout the development of an organism, cells are tuned in to their environment in order to respond effectively to any variations that may occur. The relationship between the cell and its surroundings is nurtured by signalling pathways that are responsive to specific stimuli coming from the environment or from the cell itself. The importance of signalling pathways has been highlighted by their involvement in all aspects of cellular life, and the discovery that deregulation of these pathways can potentially lead to pathogenesis. Consequently, the study of the function and mechanisms of signalling pathways is essential in understanding the evolution of human disease.

1.1 Regulation of intracellular signalling by small GTPases

Due to the broad range of their cellular functions, small GTPases are recognized as master regulators of signal transduction. Their structure and the diversity of their functions will be discussed in the first part of this introduction.

1.1.1 Small GTPases in signal transduction

The superfamily of small GTPases includes over 150 members which are grouped in at least five subfamilies based on primary sequence identity: Ras, Rho, Rab, Arf and Ran. The subfamily of each small GTPase is indicative of its function: Ras proteins mainly regulate gene expression, Rho proteins regulate cytoskeletal remodelling as well as gene expression, Rab and Sar1/Arf proteins regulate the trafficking of intracellular vesicles, and Ran proteins regulate cell cycle events such as nucleocytoplasmic transport and microtubule organization (reviewed in [1]). Further subdivisions can be made within the Ras subfamily of GTPases which includes five subgroups: classical Ras proteins (p21 Ras), Rap, M-Ras, R-Ras and Ral, which all share 40 to 50% identity with p21 Ras [2, 3]. Furthermore, Ras-like GTPases such as Rheb, Rit and Rin, can also be added to the subfamily. As GTPases within the superfamily are involved in many aspects of cellular function, any upset in their regulation may lead to diseases, such as cancer.

1.1.2 Ras GTPases and tumorigenesis

Ras subfamily members are small monomeric GTP-binding proteins (G proteins or GTPases) with molecular masses that range between 20 and 40 kDa. They are related to heterotrimeric G proteins (involved in G protein Coupled Receptor (GPCR) signalling), as well as G proteins mediating protein synthesis. The establishment of the Ras subfamily of GTP-binding proteins is historically related to the discovery of viruses that could induce tumours in mice [4]. The formation of tumours was attributed to mutated versions of genes that encoded enzymes with an intrinsic ability to hydrolyse guanine nucleotide triphosphate (GTP) into guanine nucleotide biphosphate (GDP), the process also being called GTPase activity. These viral genes were called *ras* for rat sarcoma. Cellular oncogenes were then identified in humans as mutated forms of ras proteins in some human carcinomas. Further studies of mutated *ras* genes demonstrated that they could induce proliferation and transformation of cells in culture, as well as stimulate differentiation of neuronal cells (reviewed in [1]).

The onset of cancer is caused by the disruption of the highly controlled balance between cell proliferation and cell death. In fact, deregulation of key signalling pathways can precipitate the evolution of cancer by shifting the equilibrium towards uncontrolled cellular growth. For instance, tumour cells are selected for their increased survival potential. Consequently, they can withstand death signals brought on by aberrant functions such as rapid independent proliferation and DNA damage (reviewed in [5]). As small GTPases are important components of intracellular signalling, events disrupting their normal function are regarded as potentially being tumorigenic. The observation that a murine leukaemia virus could induce tumours in new-born rodents fueled a whirlwind of research based on the oncogenic potential of *ras* genes. Subsequently, interest in the study of the function of Ras proteins and other GTPases stemmed from their ability to induce transformed cellular phenotypes when overexpressed [2, 5].

1.1.3 Structure and post-translational modifications of Ras family GTPases

1.1.3.1 Structure of small GTPases

Ras subfamily members across species share an amino acid identity of 30 to 55% [1, 2]. Like all GTP-binding proteins, Ras family members possess an intrinsic GTPase activity, which allows them to switch from a GTP-bound active state to a GDP-bound inactive state. They all share 5 consensus amino acid sequences (regions G1 to G5), that form a catalytic domain, which is essential to their function as molecular switches capable of activating downstream effectors. The G1, G4 and G5 conserved regions mainly stabilize the interaction of the G protein with guanine nucleotides GDP and GTP [6]. The G2 and G3 conserved regions are referred to as switch regions I and II respectively. As GTPases hydrolyse GTP to GDP, the two switch regions change their conformation appreciably, thus reducing the affinity of the protein for its effectors [6, 7]. Since GTPase activity is relatively low, GTPases are associated with guanine activating proteins (GAPs), which catalyse hydrolysis. Furthermore, guanine exchange factors (GEFs) promote the substitution of GDP for GTP in specific GTPases. Each GTPase possesses its own set of specific effectors which it can readily activate. The switch I region and the amino acid context in its vicinity regulate the specificity of effector binding, though in some cases, members within the same family share downstream effectors (reviewed in [8, 9]).

1.1.3.2 Post-translational modifications

Although the catalytic domain of Ras GTPases is essential for their function, cellular localization is also important. For instance, cellular localization can modulate GTPase function, as constraint of a protein to a particular cellular compartment restricts its interactions with potential effectors. Ras subfamily GTPases are membrane-associated proteins and their ability to interact stably with membranes is essential to their biological activity. Membrane association is mediated by post-translational modifications occurring in the last few amino acids of the carboxy-terminus of the proteins. These amino acids constitute a consensus sequence that includes at least one cystein (Cys) residue. The following sequences represent the conserved motifs that undergo post-translational

modifications: C-A-A-X (A: aliphatic acid, X: any amino acid), C-X-C or C-C (reviewed in [1, 10]). The modifications occur in three steps; the first one being the isoprenylation (attachment of a polyisoprene lipid) of the invariant Cys within the motif. Two classes of prenyltransferases can recognize the motif: farnesyltransferase (FTPase) and geranylgeranyltransferase (GGTase). For all Ras GTPases, the nature of the motif dictates what type of lipid will be attached. In fact, the Cys residue within a CAAX motif ending with a leucine (Leu) or a phenylalanine residue (Phe), such as in Rap GTPases, is geranylgeranylated by GGTase I. Moreover, the Cys residue in Ras proteins, which possess a CAAX motif ending with a residue other than Leu or Phe, is farnesylated by FTPase. Isoprenylation itself constitutes a signal that triggers two other post-translational modifications. In most Ras GTPases, the next step is the removal of AAX amino acids by the endoprotease Rce1. Then, post-translational modifications are resumed by carboxyl methylation of the prenylcysteine by Icmt, a methyltransferase (reviewed in [1, 11]). Post-translational modifications of the CAAX motif are essential in providing a hydrophobic portion to otherwise hydrophilic GTPases, thus allowing them to associate with hydrophobic cellular membranes such as the plasma membrane and intracellular vesicles [11].

1.2 Ras signalling

Since approximately 20 to 30% of all human tumours contain mutated forms of the prototypical GTPases of the Ras subfamily, the classical Ras proteins (Ha-Ras, K-Ras and N-Ras) have been more extensively studied compared to other Ras subfamily members [1, 12]. The focus of this section will be the function of Ras proteins, as well as their involvement in carcinogenesis. Moreover, we will discuss the function of CNK, a scaffold protein involved in Ras-MAPK signalling.

1.2.1 Ras proteins

The mammalian Ras proteins, H-Ras, K-Ras and N-Ras share a high amino acid sequence identity (85%), which accounts for their common effectors and localization. The mutated *ras* alleles found in tumours have missense mutations in residues 12, 13 or 61 of

K-Ras (85%). These mutations proved to block the catalytic activity of Ras proteins (reviewed in [12-14]). In fact, the oncogenic Ras proteins were shown to have an activity up to 300-fold higher than normal Ras [14] Moreover, *Ras* mutations are implicated in a third of all human tumours, and represent a vast majority of the mutations found in certain types of tumours such as pancreatic (90%), colorectal (45%) and lung (35%) carcinomas. Consequently, Ras proteins have become choice molecular targets for cancer therapy.

Following the molecular cloning of *ras* genes in the eighties, structural homology analyses revealed that Ras proteins were related to the alpha subunit of G proteins, suggesting an involvement in signal transduction. Indeed, Ras proteins serve as transducers of external signals such as hormones and growth factors at the plasma membrane. Following the activation of Ras by cell-surface receptors, Ras signalling can be directed down as many roads as there are Ras effectors, many of these roads leading to tumorigenesis when Ras activation is constitutive. For instance, Ras signalling regulates cell survival and cytoskeletal rearrangement through its interaction with phosphatidylinositol 3-kinase (PI3K), an important regulator of cellular growth [15]. Interestingly, most human tumours contain mutations in components of Ras or PI3K signalling, and impaired signalling in both pathways can result in very aggressive tumours (reviewed in[16]). Other Ras effectors have also been linked to cancer promoting mechanisms: activation of the Ral-GEF RalGDS can lead to uncontrolled cell cycle progression, activation of phospholipase Cε (PLC) is involved in PKC and calcium signalling, and inhibition AF6/canoe a regulator of cell-cell adhesion and cell polarity (reviewed in [12, 14, 17]). In conclusion, activation of Ras effectors can contribute to malignancy in many ways.

1.2.2 The Ras/MAPK pathway

The most studied Ras effector is Raf, a serine/threonine kinase sitting at the top of a MAPK signalling cascade. Mitogen-activated protein kinase (MAPK) signal transduction cascades have been extensively characterized as they participate in a plethora of cellular events such as differentiation, proliferation, migration and cell survival. These signalling cassettes were first observed in yeast, which utilizes 5 different MAPK pathways to

regulate mating, filamentation, osmolarity responses, cell wall remodelling and sporulation (reviewed in [18, 19]). These pathways have been conserved in all eukaryotes, and this conservation over the course of evolution denotes their key role in regulating various cytoplasmic and nuclear activities.

MAPK cascades are mostly organized in a three-kinase hierarchy which relies on protein phosphorylation for signal transmission: a MAPK kinase kinase (MAPKKK) activates a MAPK kinase (MAPKK), which in turn activates a MAPK. The formation of signalling complexes, such as MAPK, allows external signals to mediate localized cellular processes, that put in play specific effectors [20]. Hence, MAP kinase pathways act as signal insulators that can regulate and direct extracellular signals to specific locations in order to mediate appropriate responses. The classical MAPK cascades found in mammals are the ERK, JNK and p38 cascades, which are conserved among eukaryotes. The c-Jun N-terminal kinase (JNK) and p38 cascades orchestrate stress responses (reviewed in [21]). As for the extracellular signal-regulated kinase (ERK) pathway, it is involved in cellular processes such as proliferation, differentiation, cell-cycle-regulation and survival. It also regulates developmental processes such as vulval induction in *Caenorhabtitis elegans*, and eye development in *Drosophila melanogaster* [18]. Drosophila eye development will be explained in further details in section 1.4.1.2. In mammals and other eukaryotes, MAPK cascades are primarily activated by GTP-binding proteins. In the case of ERK, activation is mostly regulated by Ras proteins. Thus, I will refer to the mammalian ERK signalling cascade, as well as its counterparts found in other eukaryotes as the Ras/MAPK pathway.

Ras/MAPK signalling can be activated by various upstream receptors. Nonetheless, I will focus on activation by receptor tyrosine kinases (RTK) which are of particular interest in our laboratory. RTKs such as EGF, PDGF and the insulin receptor, are found at the cell surface. They are activated through binding of their ligand, a hormone or a growth factor, to their extracellular domain. In turn, their cytoplasmic tyrosine kinase domain phosphorylates different substrates including themselves [18]. While different sequences of molecular events lead to the activation of Ras, we will only describe one of these sequences for the purpose of this introduction. The adaptor protein Shc is recruited by binding to the

phosphotyrosine residue found on most activated RTKs, and is phosphorylated either by the RTK or a tyrosine kinase like Src. Subsequently, phosphorylation of Shc allows it to interact with another adaptor protein, Grb2, through its Src-homology 2 (SH2) domain. Given that the guanine exchange factor SOS is constitutively bound to Grb2, recruitment of Grb2 to the plasma membrane also recruits the Ras-GEF, and in turn activates Ras at the plasma membrane (a detailed description of RTK signal transduction can be found in [18, 21]).

Consequently, the MAPK cascade is launched with the first step being the recruitment of a MAPKKK, the serine/threonine kinase Raf, to the plasma membrane. There are three isoforms of Raf in mammals: Raf-1, B-Raf, and A-Raf which are ubiquitously expressed (reviewed in [22]). In model organisms, such as *Drosophila melanogaster*, there is only one homolog, DRaf, which shares the greatest sequence homology with mammalian B-Raf [23]. The interaction between Raf and Ras-GTP results in the activation of the kinase (reviewed in [22]) Upon activation, Raf sends external signals down to a specific set of MAPKK-MAPK. In fact, Raf phosphorylates dual-specificity kinase MEK1/2, which then phosphorylates a specific dual-specificity MAPK, in this case, ERK1/2 [18]. Once MAPK is activated, it stimulates cellular growth through interactions with its various cytoplasmic and nuclear effectors (reviewed in [20]).

Beyond the three-component cascade, scaffold proteins have proven to be essential for efficient signal transduction in MAPK pathways. Scaffold proteins are thought to play an important role in signal integration, and in the formation of networks which involve multiple signalling pathways [24, 25]. Concomitantly, these proteins can serve as platforms on which members of signalling cascades can interact. However, there is an increasing amount of evidence showing that interactions with scaffolds can actively modulate protein function. From the study of MAPK pathways in yeast, scaffold proteins have been found to organize signalling cascades to ensure the sequential activation of its members, to restrict signal acquisition and specificity, and to regulate the output of signal transduction [21]. The Ras/MAPK pathway is regulated by many proteins acting as scaffold proteins; one of them being kinase suppressor of ras (KSR). KSR was first isolated from genetic screens as a

suppressor of phenotypes induced by activated Ras in model organisms, *C. elegans* and *D. melanogaster* [26-29]. Sequence alignment of eukaryotic orthologs revealed that the putative kinase domain of KSR proteins is homologous to the kinase domain of Raf [29]. However, to date, there is no clear indication that KSR is in fact a kinase; see reviews for more on KSR function [24, 30, 31]. The following section will describe the function of connector enhancer of KSR (CNK), another scaffold protein involved in the Ras-MAPK pathway.

1.2.3 CNK: structure and function of a scaffold protein

1.2.3.1 Identification and structure

Such as KSR, the function of CNK has been mostly linked to the Ras-MAPK pathway. *cnk* was isolated from a genetic screen based on Ras function in *Drosophila*. Overexpression of the kinase domain of KSR in the *Drosophila* eye induces a rough eye phenotype by blocking *ras*-dependent photoreceptor cell differentiation, a process largely regulated by the Ras-MAPK pathway [32]. A genetic screen was conducted by *Therrien et al*, in order to further characterize KSR function. The screen isolated *cnk* alleles as enhancers of the KSR dominant negative (KDN) rough eye phenotype [33, 34]. Since *cnk* is recessive lethal, clonal analysis and a hypomorphic *cnk* allele were used to assess the functional relationship of CNK with the Ras-MAPK pathway. That relationship was then confirmed by the observation that a mutant *cnk* background causes the loss of R7 and outer photoreceptors in flies. Furthermore, genetic interactions with members of the pathway confirmed the positive role of CNK in Ras signalling and placed CNK function upstream or in parallel of Raf [33]. In *Drosophila*, the link between CNK and Ras signalling was also established by other studies wing and notum [35, 36]. In mammals, there are three human isoforms of CNK (CNK1, CNK2A and 2B, CNK3), and 2 rat Membrane Associated GUanylate Kinase-INteracting proteins (MAGUIN-1 and shorter MAGUIN-2), which share a rather high amino acid sequence identity with human CNK2A and 2B respectively [37]. *C. elegans*, like *Drosophila*, only has one CNK isoform, termed CNK-1 [33, 38].

From *Drosophila* to higher eukaryotes, CNK possesses multiple known conserved protein-protein interaction domains and motifs such as: a sterile α Motif (SAM), a PDZ

domain (described in section 1.3.4.1.3), SH2 interaction motifs, and SH3 interaction motifs [33]. In addition, the same sequence alignments revealed a region between the SAM and PDZ domains that is conserved among eukaryotes, the Conserved Region in CNK (CRIC). Moreover, the pleckstrin homology (PH) has proven to be required for the localization of CNK at the plasma membrane [39]. Conclusively, evidence of the implication of CNK in Ras signalling, as well as its numerous interaction domains, suggest that CNK could act as a scaffold in the Ras-MAPK pathway and possibly in other signalling pathways.

1.2.3.2 Function in Ras-MAPK signalling

Studies in *D. melanogaster*, *C. elegans* and mammals have been able to dissect many aspects of CNK function in the Ras-MAPK pathway. For instance, the use of genetics and biochemistry in *Drosophila* demonstrated that the N-terminus of CNK (SAM and CRIC domains) could integrate signals from activated Ras, and play a positive role in the activation of DRaf. Upon upstream activation by Sevenless RTK or by insulin in *Drosophila* S2 cells, CNK promotes the interaction between DRaf and KSR, thus mediating the KSR-dependent activation of DRaf at the plasma membrane [40-43]. Furthermore, siRNA against CNK2 inhibits ERK activation in rat PC12 cells after stimulation with NGF [44]. However, the positive effects of CNK on Raf activation appear to vary from one organism to another. Case in point, CNK-1 in *C. elegans* is not essential in Ras-MAPK signalling, and though CNK-1 appears to promote the activation of LIN-45 (Raf), no direct interaction of these two proteins has been reported yet [38]. In mammals, the regulation of c-Raf by CNK proteins appears to be even more complex. While early reports show that human CNK1 does not interact with Raf, recent studies demonstrate that both CNK1 and CNK2 bind to c-Raf, and positively contribute to Ras-MAPK signalling [33, 37, 45].

Besides positive regulation of the Ras-MAPK pathway, CNK was found to negatively regulate Ras signalling through inhibition of DRaf activity, as overexpression of full-length or C-terminal (amino acid 381 to 1,554, including putative SH3 and SH2 binding motifs, as well as the PH domain) CNK can block photoreceptor cell differentiation, as well as suppress an activated DRaf phenotype [33, 41]. Further

investigations identified a region located in the C-terminus of CNK that mediated its inhibitory effects on DRaf activity. This stretch of approximately 40 amino acids is called the Raf Inhibitory Region (RIR) [40]. This region is composed of two elements: the Raf Interacting Motif (RIM) which interacts with DRaf and the Inhibitory Sequence (IS) which is required for the inhibitory function of the RIR [40]. Concurrently, the SH2 domain of *Drosophila* Src homolog, Src42A, was found to bind to a SH2 binding site proximal to the RIR in a RTK-dependent manner [46]. This interaction proved to release the inhibitory effect of CNK on DRaf. Finally, it was suggested that the inhibitory function of the RIR could serve to prevent inappropriate activation of the Ras-MAPK pathway in the absence of an upstream signal. However, the differences between the various CNK orthologs suggest that their impact on Ras-MAPK signalling varies from species to species. For instance, the C-terminal RIR is only conserved in *D. melanogaster* and *Anopheles gambiae*, suggesting that a region that differs from the RIR may exert Raf recruitment in other CNK proteins.

1.2.3.3 Involvement in other signalling pathways

As mentioned previously, there are indications that the function of CNK may go beyond the Ras-MAPK pathway. Indeed, CNK1 was shown to mediate apoptosis through its association with pro-apoptotic proteins Ras-ASSociation Domain Family-1 (RASSF1) and the angiotensin II type 2 (AT2) receptor [47, 48]. CNK1 and CNK2 have also been shown to interact with the small GTPase Ral, which is involved in cellular events such as receptor endocytosis and cytoskeletal rearrangement, and its activator Ral-GDS, which is also a Ras effector [37, 49]. However, the biological significance of these interactions has yet to be determined. Furthermore, there is evidence of the implication of CNK in Rho GTPase signalling, excluding Rac or Cdc42 signalling. Two separate studies showed that human CNK1 mediate Rho-induced gene transcription through direct interactions with Rho, its activators and its effectors [49-52]. These interactions appear to regulate gene transcription, and not cytoskeletal remodelling events, such as Rho-induced stress fiber formation. However, although CNK1 does not regulate the actin cytoskeleton via Rho signalling, depletion of CNK1 in HeLa cells leads to significant changes in cellular morphology, suggesting that CNK1 may have a role in the regulation of cytoskeletal

rearrangements [44]. Further investigation of CNK function will undoutdedly help delineate its true impact on GTPase-dependent pathways.

1.2.3.4 Interaction with the actin cytoskeleton

The characterization of human CNK2 and its homologs rat MAGUIN-1 and -2 has revealed that CNK may function within complexes that regulate the actin cytoskeleton. Interestingly, rat CNK was first identified from a rat brain library via a yeast two-hybrid screen using the PDZ domains of Synaptic SCAffolding molecule (S-SCAM) as bait [53]. In fact, the C-terminus of MAGUIN-1 was shown to interact with S-SCAM, and with other PDZ domain-containing scaffold proteins: post synaptic density-95/synapse-associated protein 90 (PSD-95/SAP90) and densin-180 [53, 54]. Both S-SCAM and PSD-95/SAP90 are members of the membrane-associated guanylate kinase (MAGUK) protein family, and are associated to the actin cytoskeleton at the post-synaptic membrane of neurons. Further analysis revealed that the C-terminus of MAGUIN-1 contains a PDZ domain-interacting motif, which is absent in the shorter isoform MAGUIN-2 [53]. Thus, this C-terminal motif may be responsible for the interaction between MAGUIN-1 and PDZ domain-containing scaffold proteins. The presence of this motif in MAGUIN-1 suggests a role in the maintenance of post-synaptic membranes, which its isoform MAGUIN-2 does not share.

In neuronal cells, CNK is localized in neuronal cell bodies, neurites and synaptic plasma membranes; it also interacts with the plasma membrane of transfected CHO cells [39, 53-55] The PH domain was shown to be required for the interaction of both MAGUIN proteins with the plasma membrane, and for the recruitment of PSD-95 and S-SCAM to the plasma membrane in murine cells [39, 53]. In rat pheochromocytoma PC12 cells, CNK2 is required for NGF-mediated neurite extension, a morphological process that involves actin cytoskeleton remodelling [44]. Moreover, a yeast two-hybrid screen using human CNK2 as bait, isolated a protein, DAL-1/band 4.1B, involved in actin cytoskeleton regulation which is also required for neurite extension [44]. The cumulation of these results suggests that besides regulating the Ras-MAPK pathway, CNK may function as a regulator of actin dynamics via its interaction with membrane scaffold proteins. Interestingly, besides interacting with other proteins, MAGUIN-1 has been reported to form dimers or oligomers

under the control of the Ras-MAPK pathway [45, 54]. Such an event may create signalling platforms from which multiple protein interactions can occur, thus making CNK function quite significant in the coordination of signal transduction.

Finally, there is a significant amount of evidence that suggests that the function of CNK may go beyond the organizationtion of Ras-MAPK signalling. The next section describes the function of Rap GTPases, which, according to recent findings in our laboratory, may be functionally related to CNK.

1.3 Function and regulation of Rap GTPases

Over the years, the amount of data concerning Rap proteins has exploded. These proteins have proven to be important regulators of cellular functions such as gene transcription and cell adhesion. Interestingly, the outcome of Rap activation is strictly dictated by the nature of the stimuli, which activates specific intracellular pools of Rap proteins, as well as the cellular environment. The diversity of signals that converge towards Rap proteins suggests that Rap proteins are key regulators of cellular functions. The following sections review the function of Rap GTPases, and the nature of its regulation. Furthemore, the relationship between Ras effector signalling and Rap will be discussed in sections 1.3.1.3 to 1.3.1.5.

1.3.1 A subclass of Ras GTPases

Rap proteins are members of the Ras family of small GTPases. Human Rap proteins are encoded by the *rap1a, rap1b*, *rap2a* and *rap2b* genes, thus forming a subclass of Ras-like GTPases which share the same effectors and regulators [56, 57]. The *rap1* and *rap2* genes were first cloned based on their homology with the *Drosophila RAS3* gene, which was later renamed *Drosophila Roughened* [56]. The Rap1 and Rap2 isoforms, which share 62% identity, were found to be 53% and 46% identical to K-Ras, respectively, thus sharing many of the features of the prototypical Ras proteins, such as membrane targeting and GTP hydrolysis.

1.3.1.1 GTP-binding properties

The predicted guanine nucleotide binding properties of mammalian Rap proteins was confirmed in mammalian and insect cell lines. Biochemical analyses of Rap proteins revealed that they preferentially bind GTP and have a lower intrinsic GTPase activity than Ras proteins, although guanine nucleotide binding involves the same conserved regions as in Ras proteins [58-63]. Interestingly, Rap2 has a uniquely low sensitivity to GAPs compared to Rap1, suggesting that Rap2 acts as a slow molecular switch that mediates basal Rap activation [64]. The low intrinsic GTPase activity of Rap proteins is due to the substitution of a conserved glutamine residue proven to mediate intrinsic and GAP-induced GTPase activity in most GTP-binding proteins [56, 65]. Indeed, the glutamine residue at position 61 is substituted by a threonine residue, and interestingly, such a mutation causes oncogenic activation of Ras proteins [56, 66]. Yet, transient overexpression of Rap proteins in human cell lines did not induce transformation [67, 68]. Nevertheless, stable expression of Rap1 proteins was shown to induce DNA synthesis as well as morphological changes in cells, and to lead to the formation of non-invasive tumours in mice [68, 69].

1.3.1.2 Cellular localization

Although Rap proteins are ubiquitously expressed, they are more abundant in certain tissues and cell types such as rat brain, human neutrophils and platelets [62, 70-72]. Further analysis demonstrated that Rap1 and Rap2 were associated with a diverse array of cellular membranes in many different mammalian cell types. Indeed, fractionation experiments demonstrated that Rap proteins are tightly associated to membranes, as they were purified from detergent soluble, non cytoplasmic fractions exclusively [62, 70, 71, 73, 74]. Besides their CAAX motif being geranylgeranylated, Rap proteins harbour other molecular signals that target them to membranes [75]. Indeed, Rap2 proteins were shown to be palmitoylated like H-Ras and N-Ras, and Rap1 proteins have a lysine rich region upstream of the CAAX motif which is thought to mediate membrane association [3, 74].

Rap1 proteins have an overlapping cellular localization with Ras proteins at the plasma membrane in rat synaptosomes, human platelets, and human neutrophils [62, 70,

71]. Study of cellular localization in rat synaptosomes revealed that Rap1 proteins are abundantly expressed in the cytoplasmic region of most neuronal cell bodies, and suggests that Rap1 can bind to the plasma membrane as well as intracellular vesicles such as the endoplasmic reticulum (ER) and mitochondria [71]. Moreover, fractionation of different mammalian cell lines revealed that Rap1 proteins are associated to the membrane of subcellular compartments in which Ras proteins are virtually undetected [73]. Indeed, Rap1 proteins are bound to late endosomes and lysosomes, suggesting a role in endocytosis [76]. As for Rap2 proteins, they are tightly associated with cellular membranes, and localized to the Golgi and the ER, suggesting a role in the secretory pathway [74, 76].

1.3.1.3 Tumour-suppressor effect of Rap1a

Historically, functional analysis of Rap1 suggested that it can antagonize the transformation activity of NIH 3T3 fibroblasts infected with a virus carrying the gene *K-ras* (DT cell line). Indeed, Rap1a cDNA was isolated from a screen and the transcript was named *Krev-1* (*K-ras* revertant) for its ability to induce non-transformed "flat" cell clones in DT cells [72]. Moreover, the expression of Rap1a induces growth rate reduction and increased attachment to the substratum. Interestingly, overexpression of Rap2 has failed to suppress *ras*-induced transformation [67].

1.3.1.4 Ras effector binding

When compared to Ras, the effector region (residues 32-44) is perfectly conserved in Rap1 proteins, and differs by one amino acid in Rap2 proteins, thus suggesting that Rap proteins antagonize Ras signalling by trapping its effectors [56, 72]. However, while crystal structure and biochemical data show that Rap1 can associate with c-Raf via its effector region in vitro, there is no clear evidence that endogenously activated Rap1 can inhibit the kinase activity of c-Raf [77-79]. The most physiologically relevant interaction with Ras effectors remains the association of Rap1 with B-Raf, which was shown to promote the activation of the Ras-MAPK pathway in B-Raf expressing cells, such as PC12 cells [80-82].

1.3.1.5 Activation of ERK

Rap1 has been implicated in the activation of ERK in many cellular models, including PC12 cells. Evidence that activated Rap1 leads to ERK signalling was first obtained by inducing neuronal differentiation of PC12 cells via cAMP-mediated Rap1 activation [80]. *Vossler et al* demonstrated that cAMP-dependent protein kinase A (PKA) mediates ERK-dependent transcription via Rap1/B-Raf signalling in PC12 cells and simian fibroblasts.

Interestingly, the stimulation of PC12 cells with NGF also results in differentiation, whereas stimulation with EGF leads to proliferation [83, 84]. Moreover, NGF-induced ERK activation requires both Rap1- and Ras-dependent B-Raf activation to mediate gene transcription and cellular differentiation [81, 85]. Seemingly, NGF-induced Rap1 activation occurs after the internalization of the TrkA receptor in clathrin-coated endosomes, suggesting that Rap1 may signal from intracellular vesicles [85, 86]. In recent years, experimental data reported that NGF and cAMP can activate PKA in PC12 cells, as well as mediate Rap1 activation via a Src-dependent mechanism [87, 88]. Since NGF reportedly increases the amount of cAMP in neuronal cells, both may work together to mediate Rap1-induced neuronal differentiation [89, 90]. Moreover, the role of Rap1 in ERK activation has also been reported in other mammalian cell types besides P12 cells. In platelet precursors, sustained ERK signalling induced by thrombopoietin (TPO) requires Rap1 activation and leads to differentiation, whereas erythropoietin (EPO) induces transient ERK activation through Ras [91-94]. Likewise, Rap1 is activated by the oncogenic translocation BCR/ABL, leading to B-Raf and ERK activation in leukocytes [95]. Interestingly, constitutive Rap1 activation in myeloid progenitor cells results in constitutive ERK activation, and induces leukemias in mice [96]. All of these results suggest that the activation of Rap1, like that of Ras, contributes to ERK signalling although it is B-Raf-dependent.

The proven activation of ERK signalling by a Rap1/B-Raf complex is a world away from initial data suggesting that Rap1 activation inhibits ERK activation. For example, cAMP signalling was proven to block proliferation and ERK activation through the

inhibition of Raf-1 in mammalian cells such as PC12 cells, fibroblasts and T lymphocytes [78, 97-100]. Interestingly, Ras and Rap1 are activated in different cellular compartments in fibroblasts and neuronal cells. Indeed, EGF activates Ras at the plasma membrane, whereas Rap1 is activated exclusively within the perinuclear region of fibroblasts [101, 102]. Since receptor endocytosis seems to be necessary for growth-factor-induced activation of Rap1, Rap1 activation may be inhibited at the plasma membrane to prevent it from binding to Raf-1 and inhibiting Ras signalling [101, 102].

Thus the effect of Rap1 activation may depend on the Raf isoform available within a cell type. For instance, activated Rap1 stimulates MAPK signalling and induces cellular differentiation and proliferation in Drosophila [103, 104]. Moreover, Rap1 is thought to be involved in Ras-MAPK signalling in *D. melanogaster*, as the gain-of function mutation *Roughened* (R) induces the loss of photoreceptor cells in the adult *Drosophila* eye, a hallmark of the disruption of Ras-MAPK signalling [105-108]. Since the amino acid sequence of *Drosophila* Raf (DRaf) is closest in homology to mammalian B-Raf, the activation of MAPK signalling by *Drosophila* Rap1 may be due to the fact that the only Raf protein available is a B-Raf homolog. Interestingly, in some cases, activated Rap1 does not activate nor inhibit ERK signalling [109]. Consequently, the large amount of conflicting data suggests that the regulation of ERK signalling by Rap1 and the subsequent physiological outcomes are cell-type specific.

1.3.1.6 Signal transduction

Many different extracellular signals stimulate Rap1 activation in cell types such as fibroblasts, platelets or T-cells. In most cases, the activation of Rap1 is very rapid, suggesting that activation takes place in the vicinity of receptors [110]. Indeed, Rap1 is activated by growth factors such as PDGF and EGF, and hormones via the activation of RTKs or GPCRs, respectively [109, 111]. Moreover, cellular events such as ERK activation rely on the activation of Rap1 by intracellular cAMP [reviewed in [112]]. Phospholipase C (PLC) and the secondary messengers it generates, diacylglycerol and calcium, can activate Rap1 as well [109]. Following its activation, Rap1 interacts with proteins that harbour a Ras/Rap association (RA) domain or a Ras binding domain (RBD),

two domains that are found in many different cytoplasmic proteins (reviewed in [113]). Thus, the broad range of Rap1 activators and effectors implies that Rap1 is an important regulator of cellular functions.

1.3.2 Regulation of cell adhesion and the cytoskeleton

Over the years, Rap GTPases have been implicated in the regulation of morphological changes via cadherin- and integrin-dependent cell adhesion. Cadherins and integrins are cell-surface receptors that regulate cell-cell adhesion and cell adhesion to the extracellular matrix (ECM), respectively. The following sections focus on the role of Rap1 proteins in cell adhesion, which has been more extensively studied than the role of Rap2 proteins. In order to simplify the nomenclature, the Rap1 proteins will now be referred to as Rap1.

1.3.2.1 Regulation of the actin cytoskeleton

The ability of *rap1a* to produce "flat" non-transformed revertants in DT cells was the first evidence that Rap proteins have a role in the regulation of morphological changes [72]. Indeed, the injection of activated Rap1 in fibroblasts can induce membrane ruffling and cell spreading, while downregulating *rap1* signalling induces cell rounding [68, 114]. In the social amoeba, *Dictyostelium discoideum*, the expression of *rap1* increases cell spreading and phagocytosis, while inhibiting the ingestion of fluids by endocytosis [115-117]. These results confirmed that Rap1 could regulate biological functions that rely on the actin cytoskeleton.

Furthermore, study of the closest Rap1 homolog in *S. cerevisiae*, Bud1 (Rsr1), revealed that the small GTPase was required for proper bud site selection in yeast, suggesting a role in the establishment of cellular polarity during budding [118, 119]. Moreover, it was later established that Bud1 is regulated at the presumptive bud site by a GEF, Bud5, and a GAP, Bud2 [120-122]. The cycling of Bud1 between GTP-bound and GDP-bound states at the bud site leads to the recruitment of polarity establishment factors including the scaffold protein Bem1, the Rho family GTPase Cdc42 and its GEF Cdc24, thus leading to cytoskeletal remodelling and bud formation [123-126].

Rap1 can also mediate polarity in mammalian neurons, as Rap1 positively regulates the activation of the Rho family GTPases Cdc42 and Rac, resulting in neurite extension [127, 128]. Furthermore, the Rap1-dependent activation of Rac and Cdc42 mediates cell adhesion in epithelial cells [129, 130]. Ultimately, Rap1 appears to regulate polarity-driven cellular functions such as cell-cell adhesion and migration.

1.3.2.2 Cadherin-mediated cell adhesion

Cell-cell adhesion is primordial in the maintenance of epithelial integrity throughout development. Extensive studies of the epithelium in *Drosophila* have revealed that apical-basal polarity is created by the establishment of three types of cellular junctions: the sub-apical complex, the basally located septate junction, and the centrally located adherens junctions (reviewed in [131]). In mammals, these are called tight junctions, basal region, and adherens junctions, respectively.

The mammalian cadherin family harbours over 100 proteins, which are organized in subgroups based on their molecular characteristics. The classical cadherins are single-pass transmembrane glycoproteins that form mostly homotypic interactions in adherens junctions [132]. Cadherins dimerize in a calcium-dependent manner via extracellular cadherin repeats [133-136]. In adherens junctions, the cytoplasmic tail of the prototypical cadherin, E-cadherin, interacts with the cytoskeleton through adapter proteins of the armadillo-repeat family, such as β-catenin and α-catenin [137]. In fact, β-catenin simultaneously binds to the cytoplasmic tail of E-cadherin and to α-catenin [138-142]. Thus, α-catenin provides a link with the actin cytoskeleton as it binds to filamentous actin (F-actin), and actin-binding proteins, such as α-actinin, vinculin, zonula occludens-1 (ZO-1), and afadin (AF-6) [143-146].

The establishment of mature adherens junctions via cadherin-mediated cell adhesion is required for the formation of other types of cellular junctions [147]. Interestingly, cadherins promote the translocation of cytoplamic proteins from vesicular compartments to the membrane, and confer cellular polarity as a result [148, 149]. In fact, studies in *Drosophila* have proven that adherens junction proteins are essential for the establishment

of cell polarity during cell division [150-153]. Moreover, cadherins mediate cell sorting during embryonic tissue development [154-156]. They were also reported to be involved in the activation of Rho GTPases and RTK signalling [157, 158]. Finally, their wide range of adhesion-related functions suggests that cadherins may have a role in tumorogenesis (reviewed in [159, 160]).

1.3.2.3 Integrin-mediated cell adhesion

Integrins regulate tissue development as well as adhesion-related processes such as cell migration [reviewed in [161]]. They are cell surface receptors that function as heterodimers composed of one α and one β subunit. They have been conserved in all multicellular organisms: *C. elegans* possesses one β subunit and two α subunits, *D. melanogaster* has five α and two β, and humans possess 18 α and 8 β subunits [reviewed in [162, 163]]. As transmembrane receptors, integrins are involved in bidirectional signalling: "outside-in" and "inside-out" signalling. Their large extracellular region interacts with ECM proteins (fibronectin, laminin, collagen), cell adhesion molecules (ICAM, VCAM), or plasma membrane proteins [reviewed in [164]]. Upon binding to the ECM, integrins cluster into focal adhesions where the cytoplasmic tail of the β subunit interacts with F-actin and actin-binding proteins such as talin, filamin and α-actinin [165-167]. Integrins can regulate actin cytoskeleton dynamics via the activation of Rho family GTPases, which induce cytoskeletal rearrangements [168-170]. Furthermore, integrins can stimulate cellular proliferation and differentiation by activating ERK signalling in a RTK-dependent manner, and by activating the focal adhesion kinase (FAK) [171-174]. The regulation of integrins, or "inside-out" signalling, influences affinity, avidity and their number at the cell surface. For instance, Ras GTPases H-Ras and Rap1 can both regulate integrins depending on the cellular context (reviewed in [175]). Overall, integrins provide an important link between the environment and intracellular signalling.

The ablation of various integrin heterodimers in mice results induces defects in leukocyte function or haemostasis, as well as embryonic death (reviewed in [163]). In *D. melanogaster* and *C.elegans*, integrins have been implicated in developmental processes such as cuticle formation, cell migration and epithelial integrity, confirming their

importance in mediating cell adhesion (reviewed in [176]). Since the progression of cancer involves the loss of anchorage-dependent growth and tumour cell migration, aberrant integrin regulation may be involved in tumorogenesis.

1.3.2.4 Regulation of cell-cell adhesion

Rap1 has been shown to regulate many morphological processes involving cell-cell adhesion in eukaryotes. In *Drosophila*, loss of function of *R* leads to embryonic lethality due to severe morphogenetic defects including failure to undergo gastrulation [108, 177]. *R* loss of function in the *Drosophila* ovary and eye induces aberrant migration and cell shape, which cause tissue degeneration [108, 177]. In *Drosophila*, cells that do not express *R* fail to establish *de novo* adherens junctions with surrounding cells after cytokinesis, causing them to aberrantly migrate within the epithelium [178]. Moreover, the actin-binding protein Canoe, an homolog of human AF-6, mediates embryonic dorsal closure in Drosophila in a Rap1-dependent fashion [179]. Interestingly, AF-6 knockout mice fail to complete embryogenesis due to severe defects in cell-cell adhesion and epithelial polarity [180].

Many studies conducted in mammalian systems have proven that Rap1 signalling stimulates cell-cell adhesion by positively regulating homophilic cadherin interactions. Firstly, both Rap1 and its activator, DOCK4, were shown to restore adherens junctions in mouse osteosarcoma cells [181]. Secondly, Rap1 signalling regulates E-cadherin-mediated cell-cell adhesion by recruiting E-cadherin molecules to establish mature adherens junctions in epithelial cells [129, 182, 183]. Thirdly, Rap-GEF C3G was shown to interact with the cytoplasmic tail of E-cadherin and to activate Rap1 at nascent adherens junctions, [129, 183]. Interestingly, the ablation of C3G causes embryonic lethality in mice due to severe defects in epithelial cell adhesion [184]. Finally, in the mammalian vascular endothelium, cAMP-activated Rap1 promotes tightening of cellular junctions via the enhancement of cell-cell adhesion mediated by vascular endothelial cadherin (VE-cadherins) at adherens junctions [185, 186]. The scaffold protein MAGI-1/BAP-1, which interacts with the α- and β-catenin, was shown to be required for this process [187].

The studies described above suggest that Rap1 signalling influences cell-cell adhesion via the regulation of membrane proteins within adherens junctions. However, no clear mechanism of Rap1 induced cell-cell adhesion has been described to date. Nonetheless, a recent study proposes a model for Rap1 activation. Indeed, *Balzac et al.* shows that Rap1 is activated upon adherens junction disassembly and E-cadherin endocytosis in mammalian cell lines. Rap1 and endocytosed E-cadherin colocalize at the perinuclear region, and Rap1 is subsequently downregulated when E-cadherin molecules are recycled to the plasma membrane [188]. In conclusion, this suggests that Rap1 activation is regulated by E-cadherin internalization, thus Rap1 appears to be a sensor of adherens junction integrity.

1.3.2.5 Regulation of adhesion via integrins

Unlike Ras proteins, which regulate positively or negatively integrins in a cell-specific manner, Rap has proven to be an unconditional activator of integrins (reviewed in [175]). Cells of the human haematopoietic lineage have been instrumental in characterizing the regulation of integrins by Rap1. Early on, Rap1 was found to be the most abundantly expressed membrane-bound GTPase in human platelets, and to mediate hormone induced platelet aggregation and spreading [62, 189, 190]. In platelet precursors, Rap1 not only stimulates differentiation, but also regulates positively the affinity of the αIIbβ3 integrin to fibrinogen [191, 192]. Lessons can also be learned from leukocytes in which haematopoietic cytokines stimulate Rap1-dependent enhancement of β1 integrins, thus regulating survival, trafficking, targeting and transendothelial migration [114, 193, 194].

The most significant advances in understanding the role of Rap1 in the regulation of integrins have been made by studying the various adhesion-related events required for the proper function of T lymphocytes. In order to ensure immune surveillance and appropriate immune responses, T cells roll passively along blood vessels in search of cells presenting foreign antigens. Upon activation by extracellular stimuli, T cells undertake three key adhesion-related processes: extravasation (arrest), migration through tissues, and the formation of an immunological synapse (adhesion with an antigen presenting cells (APC)) [195-197]. The integrin family member lymphocyte function-associated antigen 1 (LFA-1

or αLβ2) is essential in mediating all of three of these steps [196, 198, 199]. Interestingly, expression of activated Rap1 in T cells increases the clustering of β1 and β2 integrins [200]. Furthermore, Rap1 induces LFA-1-mediated cell adhesion, and regulates TCR-mediated adhesion at the immunological synapse where it colocalizes with LFA-1 [201, 202]. Moreover, Rap1 regulates LFA-1 avidity and affinity via its effector regulator of adhesion and cell polarization (RAPL) upon cytokine stimulation and TCR activation [203]. Indeed, RAPL colocalizes with LFA-1 at the leading edge of migrating T cells and where it strongly polarizes LFA-1 distribution [203]. Besides RAPL, another Rap1 effector Rap1-GTP-interacting adaptor molecule (RIAM) regulates integrin-mediated cell adhesion, as well as actin dynamics via its interaction with adapter proteins of the actin cytoskeleton profilin and ENA/VASP [204] Furthermore, Rap1 regulates transendothelial migration of leukocytes by mediating integrin activation, as well as establishing cell polarity [205].

A model for integrin activation and recycling to the plasma membrane was established in T lymphocytes. This model proposes that upon T-cell stimulation, Arf GTPase signalling regulates integrin recycling from endosomes, while Rap1 regulates the activation within the intracellular structures [206]. Moreover, a recent study suggests that Rap1 provides a link between cell-cell adhesion and adhesion to the ECM through the regulation of endosomal recycling of cadherins and integrins, as the disassembly of adherens junctions activates Rap1 and stimulates integrin-mediated adhesion to the ECM [188]. Overall, Rap1 regulates integrin activation by inside-out signalling, and establishes cellular polarity which is essential for cellular processes such as migration.

1.3.3 Regulation

The localization of Rap1 activation is considered to be an important factor in mediating Rap1-dependent cell polarity. The activation of Rap1 occurs through a broad range of stimuli, which are thought to target specific intracellular pools of Rap1. The following sections present the main GAPs and GEFs that regulate Rap1 and polarize its activation within a cell.

1.3.3.1 GTP-activating proteins

Since Rap1 has a very low GTPase activity, the cycling of Rap1 between GTP and GDP is tightly regulated by GAPs. Rap1GAP, mostly expressed in the brain, is the first Rap1-specific GAP to be isolated [207-209]. Both Rap1GAP and Rap1GAPII are targeted to the plasma membrane by interactions with the regulatory subunits of GPCRs Gαi and Gα0, and control Rap1-mediated ERK signalling [210-212]. The other major class of RapGAPs is the signal-induced proliferation associated gene-1 (SPA-1) family. SPA-1 GAPs are PDZ domain-containing proteins, and associate with actin-binding scaffold proteins such as PSD-95 at the post-synaptic membrane of neuron [213, 214]. For example, studies in T lymphocytes show that SPA-1, a GAP for both Rap1 and Rap2 is recruited to the actin cytoskeleton by actin-binding proteins AF-6 and α-actinin [215, 216]. These interactions are thought to inhibit Rap-mediated cell adhesion in resting T-cells and to titre Rap1-GTP at the immulogical synapse [215, 217]. Conclusively, GAPs are recruited to cell surface receptors and to the actin cytoskeleton to regulate negatively specific pools of Rap1.

1.3.3.2 Guanine exchange factors

Rap GTPases are activated by many cellular components, from cell surface receptors to second messengers. It has become clear that specific pools of Rap1 are activated to mediate functions that are often cell-type specific. The specificity of the activation of Rap1 is mediated in part by its GEFs, which are themselves activated by distinct pathways. All Rap GEFs possess a GEF domain homologous to CDC25, a yeast GEF, and a Ras exchange motif (REM) which offers a platform for guanine nucleotide exchange [218, 219]. However, Rap GEFs possess distinct domains that mediate their specific activation. All the known activators are described in Figure 1 [220-222]. The following describes the main subclasses of Rap1-GEFs: C3G, CalDAG-GEF and Epac. PDZ-GEF will be described in section 1.3.4.

Figure 1. Guanine exchange factors for Rap
C3G: Crk SH3-domain-binding GEF; CalDAG-GEF: Calcium Diacyl Glycerol-Guanine nucleotide Exchange Factor: Epac1/2: Exchange protein directly activated by cAMP: Repac: Related to Epac: PDZ-GEF1/2: PSD-95/Dlg/ZO-1 -Guanine nucleotide Exchange Factor: PLC: Phospholipase C: BCAR-3: Breast cancer antiestrogen resistance 3: DOCK4: Dedicator of cytokinesis. Domains are also listed. PRR: SH3 interacting domain; REM: Ras-exchange motif; RasGEF: catalytic domain: EF: Helix-Loop-Helix calcium binding: C1: diacyl glycerol binding: DEP: Dishevelled/Egl-10/Pleckstrin: cNMP: cyclic nucleotide monophosphate binding; RA: Ras/Rap1 interacting: PDZ: PSD-95/Dlg/ZO-1; PLCX/Y: Phopholipase Cx/y domain: C2: calcium-triggered phospholipid-binding module; SH2/3: Src homology: DHR-1/2: Dlg Homology Repeats

Figure 1: Rap GTPase activators

1.3.3.2.1 C3G

C3G (Crk SH3-domain-binding GEF) was the first Rap GEF to be identified; it contains a proline-rich region which interacts with the SH3 domain of members of the Crk adaptor protein family [223]. Crk recruits C3G upon RTK, TCR or cytokine receptor activation, and mediates C3G activation via tyrosine kinases at the plasma membrane or in intracellular compartments [78, 193, 221, 224, 225]. Finally, the activation of C3G by cell surface receptors positively regulates Rap1-dependent ERK signalling [103, 226-228]. However, C3G is also recruited at nascent adherens junctions where the C3G/Crk complex regulates Rap1-dependent cell-cell adhesion [129, 183].

1.3.3.2.2 Calcium and DAG regulated-GEFs

CalDAG-GEF family members are GTPase activators that harbour a diacylglycerol (DAG)-binding domain (C1) and a calcium-binding domain (C2) [229]. The family counts 4 human isoforms, but only CalDAG-GEFI and III have been reported to activate Rap GTPases [230, 231]. CalDAG-GEFI regulates Rap1-dependent integrin-mediated cell adhesion and aggregation in platelets [232, 233]. Moreover, CalDAG-GEFI is required for Rap1-dependent ERK signalling from GPCRs in neurons, suggesting that CalDAG-GEFs are activated by the GPCR-dependent activation of phospholipase C (PLC), which generates calcium and DAG as bi-products [234]. Interestingly, one GEF domain-containing PLC isoform, PLCε, shows specific activity toward Rap1, and is regulated by small GTPases, Ras, Rap1 and Rho [235, 236]. This suggests that PLC isoforms may directly and indirectly mediate Rap1 activation.

1.3.3.2.3 Epac (exchange protein directly activated by cAMP)

Intracellular cAMP activates Epac proteins by binding to cyclic nucleotide monophosphate (cNMP)-binding domains, which inhibit the catalytic GEF domain in the absence of cAMP [237, 238]. Mammalian Epac1 has one cNMP domain and Epac2 has two, while the atypical Repac (related to Epac or MR-GEF) has none, suggesting that it may be constitutively active [239, 240]. Many hormones and agonists increase intracellular

cAMP levels via GPCR activation, thus regulating cAMP-dependent activation of Rap1 in mammalian cells (reviewed in [221]). While, C3G regulates PKA-dependent cAMP-mediated Rap1 activation, Epacs mediate Rap1 activation independently of PKA (reviewed in [112]). Both Epac1 and Epac2 have a Dishevelled, Egl-10, Pleckstrin (DEP) domain, which targets them to the perinuclear region [239, 241]. All three mammalian Epacs possess a RBD, suggesting that Ras family GTPases may regulate their activation. Epacs mediate the activation of Rap1 by cAMP, but they do not necessarily mediate Rap1-dependent ERK activation, as cAMP activation of Rap1 was reported to occur independently of ERK signalling in various cell-types [109, 242]. Moreover, Epacs also regulate Rap1-mediated cell adhesion by stimulating cell-cell contacts within the vascular endothelial barrier [185, 186].

1.3.4 PDZ-GEF

PDZ-GEFs are guanine exchange factors that specifically activate Rap GTPases, and they are conserved among eukaryotes. PDZ-GEFs regulate various cell adhesion processes in eukaryotes via the activation of Rap GTPases. The next sections describe the structure and the function of PDZ-GEFs in mammals and simpler organisms, focussing mainly on *D. melanogaster*.

1.3.4.1 Protein structure

Like the other Rap-GEFs, PDZ-GEFs possess a Cdc25-like GEF domain and a REM domain that both meditate the nucleotide exchange within Rap GTPases [243]. PDZ-GEFs are homologous to Epacs as they both harbour at least one cNMP domain [243]. However, their RA and PDZ domains set them apart from Epacs. This section reviews the reported functions of the various domains and conserved regions that make PDZ-GEFs unique.

1.3.4.1.1 Regulation via the cNMP domain

The human PDZ-GEF family consists of 4 proteins: PDZ-GEF1 (RapGEF2, RA-GEF1, CNrasGEF) with one cNMP domain, PDZ-GEF2 (RapGEF6, RA-GEF2, nRapGEP) and its splice variants, 2A and 2B, which have 2 cNMP domains [243]. Unlike Epacs, it is

not yet clear if the cNMP domain of PDZ-GEFs actually binds cyclic nucleotides like cAMP or cGMP. The cNMP domain of PDZ-GEFs is described as atypical, since it is similar to the cAMP- and cGMP-binding domains of PKA/Epacs and protein kinase G (PKG), respectively, although the conservation of essential motifs is not maintained [230, 244-246]. In Epacs, the cNMP domain sterically blocks access to the GEF domain, binding to cAMP changes the conformation and allows the GEF domain to interact with the REM and the nucleotide binding sites of Rap1[238, 239]. No mechanism of auto-inhibition has been reported as of yet for PDZ-GEFs, though the activity of PDZ-GEF1, and not PDZ-GEF2, is enhanced when its cNMP domain is removed [243, 246]. Despite the lack of conservation of its cNMP, PDZ-GEF1 was reported to mediate cAMP-dependent Ras/B-Raf association in human epithelial kidney cells, while it activates Rap1 independently from cAMP [245, 247]. However, studies conducted in vitro as well as in murine fibroblast cell lines demonstrate that the cNMP domains of PDZ-GEF1 and PDZ-GEF2 do not respond to small nucleotides such as cAMP or cGMP [243, 248, 249]. The same is true in *D. discoideum*, whose PDZ-GEF homolog, GbpD, does not respond to cAMP or cGMP either [250]. Furthermore, one study shows that PDZ-GEFs do not have any effect on Ras in the absence or presence of cAMP [243]. Consequently, the cAMP-mediated PDZ-GEF1-dependent activation of Ras may be cell-type specific, and the mechanism by which PDZ-GEFs are activated is clearly cAMP-independent, and remains to be identified.

1.3.4.1.2 Recruitment via the RA domain

Early studies of human and nematode PDZ-GEF1 revealed that the RA domain mediates binding to activated Rap1 specifically [248]. Indeed, the deletion of critical residues within the RA domain of PDZ-GEF abolishes the association with Rap1, and reduces its GEF activity toward Rap1 *in vivo* [249]. Moreover, the RA domain of PDZ-GEF1 mediates its translocation from the cytoplasm to the perinuclear region upon expression of activated Rap1 in COS-7 and Rat-1 fibroblasts, suggesting that PDZ-GEF1 may regulate positive feedback at the perinuclear region [249]. However, the RA domain of PDZ-GEF2 seems to regulate its function differently as it weakly binds Rap1 in a GTP-independent manner [251]. Interestingly, the RA domain of PDZ-GEF2 interacts with another GTPase, M-Ras, in a GTP-dependent manner [251]. M-Ras is part of the R-Ras

subgroup of Ras-like GTPases, whose members (R-Ras, TC21, M-Ras) respond to Ras regulators and mediate cell proliferation and integrin-mediated cell adhesion (reviewed in [3]). The interaction with M-Ras-GTP appears to translocate PDZ-GEF2 from the cytoplasm to the plasma membrane, where it enhances Rap1 activation [251]. Conclusively, the RA domain differentially regulates the localization of PDZ-GEFs, and targets them to specific intracellular pools of Rap1.

1.3.4.1.3 Protein interactions via PDZ domains

The PDZ-GEF proteins stand out among Rap-GEFs with their PDZ domains which are known to mediate protein interactions. The name PDZ was inspired from the three proteins that helped define the domain, PSD95, Dlg, and ZO-1 [252]. This domain of approximately 90 amino acids is arranged in a globular structure that interacts with specific C-terminal or internal motifs, as well as lipids and other PDZ domains (reviewed in [253]). PDZ-containing proteins organize large protein complexes at specific cellular locations via the formation of PDZ domain-dependent protein interactions. These scaffold proteins not only organize signalling networks, but they can also regulate them. Such is the case of PSD95, which regulates the activity of the NMDA receptor and the K+ channel at the postsynaptic membrane of neurons by mediating receptor clustering via PDZ-dependent interactions [254, 255]. Moreover, PDZ-containing proteins are involved in the transduction of RTK signalling, the trafficking of membrane and intracellular proteins, as well as the establishment of epithelial polarity (reviewed in [253, 256]).

The PDZ domain of PDZ-GEF1 reportedly binds the C-terminus of the GPCR β1 adrenergic receptor (β1-AR), and mediates cAMP-induced Ras activation at the plasma membrane [247]. Moreover, PDZ-GEF1 is targeted to the plasma membrane via its PDZ domain at cell-cell contacts, where it appears to colocalize with membrane proteins β-catenin and ZO-1 in epithelial cells [245, 257]. Besides possessing PDZ domains, the mammalian isoforms PDZ-GEF1, PDZ-GEF2 and PDZ-GEF2A all have a PDZ-binding motif at their C-terminus [243]. The SAV motif corresponds to the S/T-X-V consensus (where X is any amino acid) from the first class of PDZ-binding motifs which are recognized by proteins such as PSD-95 and Erbin [253]. Protein interaction studies

demonstrate that the PDZ domains of MAGUK family membrane scaffold proteins S-SCAM and MAGI-1 interact with the C-terminus of PDZ-GEF1 [258, 259]. Furthermore, mass spectrometry identified PDZ-GEF1 along with an S-SCAM isoform within as β-catenin-containing protein complex in bovine brain cytosol [257]. The interaction between PDZ-GEF1 and β-catenin was further confirmed by immunoprecipitation in epithelial cells [257]. In conclusion, PDZ-GEFs are recruited within protein complexes via their PDZ domains and/or PDZ-binding motif, where they may regulate signal transduction from cell surface receptors, as well as influence cell adhesion.

1.3.4.1.4 Ubiquitination

Among their conserved domains, PDZ-GEF proteins harbour consensus motifs that mediate various protein interactions. Early in the characterization of PDZ-GEFs, one group highlighted two conserved PY motifs (P-P-X-Y, where X is any amino acid) in mammalian PDZ-GEF1 that could potentially bind the E3 ubiquitin ligase Nedd4 [245]. The interaction between at least one of the multiple WW domains of Nedd4 and the PY motifs of human PDZ-GEF1 was later proven *in vitro* and *in vivo* [260]. *Pham and Rotin* were also the only ones to show that human PDZ-GEF1 is ubiquitinated mostly via Nedd4 activity, and partially degraded by the proteasome in human epithelial cells. Moreover, Nedd4-mediated ubiquitination of PDZ-GEF1 is dependent on the RA domain, suggesting that binding to either Rap or Ras GTPases regulates the stability of PDZ-GEF1 in mammalian cells [260].

1.3.4.2 Biological functions of the PDZ-GEF/Rap pathway

Although studies of mammalian PDZ-GEFs have provided valuable insight on their role as activators of Rap and Ras GTPases and on their interactions with proteins such as MAGUK family members and M-Ras in neuronal and epithelial cells, no PDZ-GEF-dependent biological functions have emerged from studies in mammalian systems. Luckily, the study of PDZ-GEF proteins in model organism has been useful in defining the biological functions that they regulate. While mammalian PDZ-GEF proteins stem from 2 different genes that are either expressed ubiquitously (PDZ-GEF1) or primarily in the brain (PDZ-GEF2), there is only one PDZ-GEF gene in lower organisms such as *C.elegans* and

D. melanogaster. Nevertheless, the existence of splice variants for these genes suggests that different PDZ-GEF isoforms exert different functions within lower organisms as well.

1.3.4.2.1 Regulation of cell polarity and cell adhesion in Dictyostelium discoideum

The Rap1 homolog of *D. discoideum* is essential in growth and viability, and regulates actin cytoskeleton remodelling in response to extracellular signals [115, 116, 261]. *D. discoideum* are small motile eukaryotes, and their unicellular form shares many similarities with the cell morphology and biology of cells found in multicellular organisms such as neutrophils. Upon starvation, the amoeba uses chemotaxis, an evolutionarily conserved mechanism, to aggregate into a multicellular organism [262]. Chemotaxis involves cAMP-dependent cGMP signalling that is regulated by cGMP binding proteins (Gbp), including the atypical Rap1-GEF, GbpD, which does not bind neither cAMP nor cGMP [263-265]. The domain architecture of GbpD identifies it as the most likely homolog of PDZ-GEF2 in the social amoeba, as GbpD harbours a REM, a GEF domain, and two cNMP domains [264]. Functional studies of GbpD reveal that this PDZ-GEF2 ortholog regulates cell polarity and cell adhesion. On one hand, GbpD overexpression induces a flat cell morphology due to increased substrate adhesion, and inhibits chemotaxis [250]. On the other hand, *gbpD* null mutants exhibit a very polarized cell shape, loss of substrate adhesion and increased chemotaxis [250]. Furthermore, the serine/threonine kinase, Phg2, was isolated as a Rap1 effector regulating GbpD/Rap1-dependent cell adhesion [265].

1.3.4.2.2 Regulation of epithelial integrity in Caenorhabtitis elegans

C.elegans has also been used in one study as a model in which to study the function of PDZ-GEF. There is one single PDZ-GEF gene in *C.elegans*, *pxf-1*, whose transcripts are alternatively spliced to give rise to three distinct isoforms: PXF-1-A and PXF-1-B have two cNMP domains, while PXF-1-C has one [266]. GFP reporter constructs for the *pxf-1* promoter reveal that PXF-1 isoforms are expressed strongly in hypodermal cells and the gut from early embryonic development to larval stages. Interestingly, the expression of PXF-1 in the pharynx peaks during larval molting, suggesting a dynamic regulation of its expression [266]. Worms that are homozygous for a deletion of the *pxf-1* locus exhibit a pleiotropic phenotype defined by the gradual loss of epithelial integrity due to defects in

cuticle secretion and molting [266]. The same phenotypes are observed in *rap-1* null mutants, and animals missing one allele of both *rap* genes, *rap-1* and *rap-2*. Subsequent hypodermal degeneration and lethality in late larval stages and early adulthood in *pxf-1* null mutants can be rescued by the expression of activated Rap1, confirming that the regulation of epithelial integrity is dependent on the PDZ-GEF/Rap pathway [266]. Moreover, homozygous *pxf-1* mutant hermaphrodites produce a very small progeny that rarely reaches adulthood, thus demonstrating that there is a maternal contribution of PXF-1 to developing embryos [266]. Eventually, egg laying drastically stops in homozygous mutant adults due to gonad degeneration, leading to death induced by a burst vulva, commonly known as "bag of worms" [266]. These results lead the authors to propose that PDZ-GEF/Rap signalling regulates both gonad integrity and polarized cuticle secretion from the hypodermis.

1.3.4.3 Regulation of cell adhesion in *Drosophila melanogaster*

The study of *Drosophila* PDZ-GEF, also known as Dizzy or Gef26, has been valuable in deciphering the biological pathways regulated by PDZ-GEF. The Drosophila *pdz-gef* gene was first annotated by computational analysis as CG9491 and produces at least two transcripts of 7.5 and 5.5 kb each, which are expressed in all developmental stages from the embryo to the adult [267]. At the amino acid level, dPDZ-GEF, which harbours one cNMP domain, shows about 52% sequence homology to human PDZ-GEF1, and targets Rap1 specifically. *pdz-gef* null mutants show significant lethality, as adult escapers are recovered at a very low frequency of approximately 0.01% [267]. Adult *pdz-gef* null escapers exhibit many developmental defects such as small and rough eyes, wings rolled downward with frayed margins, and sterility in both males and females [267, 268]. The following sections describe the biological processes that require dPDZ-GEF activity, as demonstrated by the four studies that have been published as of yet on dPDZ-GEF.

1.3.4.3.1 Regulation of MAPK signalling in eye development

The role of dPDZ-GEF in eye development was first demonstrated by the fact that flies with reduced dPDZ-GEF expression show a slight rough eye. Tangential adult eye sections reveal that *pdz-gef* null ommatidia can miss up to 4 of the 8 photoreceptor cells normally found in wild-type ommatidia, and are sometimes fused together [267]. Moreover,

eye-specific overexpression of dPDZ-GEF also induces a rough eye phenotype, though, in this case, it is due to abnormal clustering of ommatidia as well as increased numbers of photoreceptor cells within some ommatidia [267]. This phenotype is readily rescued by partial *R* downregulation. These results suggest that beyond playing a role in eye development, PDZ-GEF/Rap1 signalling is also involved in photoreceptor cell specification, a process that is largely regulated by the Ras-MAPK pathway [106]. In this light, *Lee et al.* demonstrated that increased photoreceptor number and aberrant ommatidium morphology due to dPDZ-GEF overexpression could be suppressed by DRaf, dMEK or dMAPK dosage reduction. Although Ras is not a functional target for dPDZ-GEF, overexpression of the GEF increased the expression of rhomboid, a transcriptional target of EGFR-induced MAPK signalling [267]. Moreover, among their many developmental defects, *pdz-gef* null mutants have a rolled down wing phenotype and wing vein defects, phenotypes that are reminiscent of those found in *rolled* (*Drosophila* MAPK) mutants [267, 268]. Hence, these results support the hypothesis that dPDZ-GEF regulates eye development, and possibly wing development, via MAPK signalling.

1.3.4.3.2 Regulation of cell-cell adhesion in gonad development

Besides regulating eye development, dPDZ-GEF plays a role in gonad development in both female and male flies as *pdz-gef* null mutant flies are sterile [267, 269]. *Lee et al.* have shown that female flies progressively undergo significant degeneration of their ovaries. Since dPDZ-GEF is strongly expressed in both nurse cells and follicle cells in developing ovarioles, PDZ-GEF is thought to regulate ovary development via Rap1 signalling [267]. Furthermore, PDZ-GEF is also expressed in spermathecae, which are female organs for long-term sperm storage. Alleles that are either null for *pdz-gef* or that exhibit strong dPDZ-GEF loss-of-function lose the protein expression in spermathecae, and harbour supernumerary spermathecae [268]. Interestingly, the PDZ-GEF loss-of-function phenotype in spermathecae is exacerbated by the reduction of either Rap1 or Rap2-like (Rap2l) protein expression, while overexpression of E-cadherin appears to rescue the supernumary spermathecae phenotype [268].

dPDZ-GEF also regulates testis development in males. For instance, germline stem cells (GSCs) and somatic stem cells (SSC), which maintain spermatogenesis, are either reduced in number or completely lost in *pdz-gef* null mutants and strong loss-of-function mutants [269]. Moreover, downregulation of *R* or *rap2l* expression enhances the stem cell loss phenotype in *pdz-gef* mutants. In mutants, stem cells drift away from the hub, a cluster of 12 somatic cells which defines the stem-cell niche and regulates their self-renewal via the JAK/STAT pathway [270]. Interestingly, PDZ-GEF/Rap signalling within the hub regulates the formation of E-cadherin-mediated adherens junctions which ensure the anchorage of stem cells to their niche [269]. Thus, it is reasonable to say that PDZ-GEF/Rap signalling regulates gonad development via E-cadherin-mediated cell adhesion in *Drosophila.*

1.3.4.3.3 Regulation of migration in embryonic macrophages

Recently, the function of PDZ-GEF was described in migrating embryonic macrophages. Indeed, in the *Drosophila* embryo, *pdz-gef* mutant macrophages failed to migrate to specific locations during the course of development and exhibited small cellular protrusions, suggesting that cell motility and substrate adhesion are impaired in these mutants [271]. Furthermore, PDZ-GEF overexpression in macrophages also results in slow migration, but in this case, impaired motility is due to increased Rap1-dependent adhesion of cellular protrusions [271]. Interestingly, nor PDZ-GEF or activated Rap1 overexpression leads to aberrant cell morphology in macrophages that do not express the integrin β gene, *myospheroid* (*mys*, βPS). These results suggest that PDZ-GEF/Rap signalling requires βPS in order to regulate cell adhesion and cell morphology in migrating macrophages.

In conclusion, PDZ-GEF appears to play an important role in the regulation of developmental processes that require cytoskeletal rearrangements. However, the sudy of PDZ-GEF is still in its infancy as important aspects of its function, such as its mode of activation, remain undefined. In time, further study of PDZ-GEF in mammals and simpler organisms will provide much more insight in the function of this Rap-GEF.

1.4 Drosophila melanogaster

Within a hundred years, the fruit fly has become more than a simple tool in the study of genetic inheritance, but has also become a model in the study of human diseases such as developmental and neurological disorders and cancer. The following sections describe the rationale behind using *Drosophila* as a model and the various approaches that can be used in order to study human disease.

1.4.1 A model organism

D. melanogaster has proven to be a very useful animal model in the dissection of signalling pathways. For instance, fruit flies are easily maintained and handled in a laboratory setting, and their short life cyle (10 days at 25°C) makes for quicker characterization of mutant phenotypes. Furthermore, there is less redundancy within the pool of genes available in *Drosophila* than within mammalian genomes, thus the phenotypic effects of gene mutations can be assessed more efficiently. Finally, the high standing of *D. melanogaster* among model organism is partly due to the impressive amount of research tools that have been generated and made available over the years. These tools range from computerized databases of genomic and proteomic information to collections of tools such as genetic stocks, cell lines and antibodies (reviewed in [272]). Moreover, an impressive number of techniques such as the generation of transgenic flies and genetic screens have been mastered over the years in order to effectively probe the *Drosophila* genome for insight on gene function. Consequently, the simple fruit fly has evolved into a powerful system in which to study the function of genes during normal development and disease.

1.4.1.1 Study of human disease

The convergence of molecular biology and genetics has provided a unique opportunity for the study of gene function and regulation in organisms such as the fruit fly. Over the years, it has become clear that many of the genes involved in classic developmental processes have been conserved throughout evolution. In the last few years, comparative genomic analyses have been conducted using the entire *Drosophila* genome

sequence and all the human genes that possess at least one mutant allele involved in human disease, as listed by the Online Mendelian Inheritance in Man (OMIM) database [273-275]. These genomic studies revealed that approximately 75% of human disease genes were conserved in the fruit fly, suggesting that the functional information obtained for any of these genes in *Drosophila* can be readily applied in vertebrates.

The conservation of genetic pathways among various species is part of the rationale behind the use of simple animal models in characterizing the role of human genes. Moreover, functional studies which provided important insight on the impact of these genes on developmental processes and human diseases have been conducted in *Drosophila* (reviewed in [276]). For instance, the use of the fruit fly as a model has been important in defining cancer pathways such as Ras-MAPK and Target of Rapamycin (TOR) which can be transposed to human development (reviewed in [277]).

1.4.1.2 Signalling pathways in *Drosophila* organogenesis

Instead of strictly relying on immortalized or primary cell cultures, the use of model organisms allows researchers to study signalling pathways in the whole animal as well as in specific tissues. For instance, *Drosophila* imaginal discs can be used to study signalling pathways. Imaginal discs are cellular epithelia formed by cell populations that are specified early in embryogenesis. Over the course of development, imaginal discs undergo cellular differentiation and proliferation to give rise to adult appendages such as the legs, antennae, eyes and wings. Thus, *Drosophila* organogenesis can be used as a system to study signalling pathways. The following two sections describe the signalling pathways that regulate the patterning of *Drosophila* eye and wing imaginal discs, which are of interest for the purpose of this research project.

1.4.1.2.1 Photoreceptor cell differentiation

The Drosophila compound eye is composed of approximately 800 hexagonal cell clusters called ommatidia. Each ommatidium contains eight photoreceptor (R1 to R8) cells, four cone cells, 2 primary pigment cells and 12 accessory cells (bristle, secondary, and tertiary pigment cells) that are shared with adjacent ommatidia [278]. Cellular

differentiation starts in the eye disc of the third larval instar, and is characterized by an apical constriction of the epithelium, the morphogenetic furrow, which sweeps across the tissue from the posterior to the anterior edge. The sequence of events that follow has been extensively reviewed, the following resumes it briefly [279, 280]. The onset and the progression of the furrow are regulated by the secreted morphogen Hedgehog (Hh), which promotes photoreceptor cell differentiation by inducing cell-cycle exit in cells located ahead of the morphogenetic furrow. Subsequently, Hh signalling stimulates the expression of the proneural gene, *atonal*, which becomes restricted to groups of cells termed proneural clusters. In turn, the expression of *atonal* activates Notch which restricts Atonal expression to one cell per prospective ommatidium, giving rise to the R8 photoreceptor cell. Subsequently, the founder R8 cell initiates the recruitment of the seven other photoreceptors by secreting Spitz, a ligand that specifically activates the *Drosophila* homolog of EGFR (DER), and inducing RTK signalling in adjacent cells via Ras [281, 282]. Impaired Ras-MAPK signalling disrupts the differentiation of photoreceptor cells, and induces a rough eye phenotype in the adult which can be used in genetic screens in order to find gene alleles that modify this phenotype.

1.4.1.2.2 Patterning of the anterior wing margin

The adult *Drosophila* wing is composed of three main regions: the blade, the hinge and the wing margin. Early in wing development, two organizing centers are defined by the anteroposterior axis (A/P) and the dorsoventral axis (D/V). The D/V axis is defined as the boundary between Apterous-expressing and non Apterous-expressing cells, and the A/P axis is defined as the boundary between Engrailed-expressing and Engrailed non-expressing cells [283]. Patterning of the wing disc occurs early in larval development as Apterous and Engrailed regulate the expression of the wing selector protein Vestigial in the prospective wing [284]. For instance, Apterous stimulates the expression of Serrate, a Notch receptor ligand, and induces Notch signalling within the D/V and A/P boundaries, which in turn activate Vg expression [284, 285]. In the third larval instar, Notch signalling is restricted to the D/V boundery cells and depends on the expression of ligands, Serrate and Delta, by cells flanking the D/V axis [285]. Notch signalling induces the expression of Cut, a cell fate determinant, as well as the morphogen Wingless within the D/V boundary.

Subsequently, secreted Wg induces Serrate and Delta expression in flanking cells, thus exerting a positive feedback loop for Notch signalling (reviewed in [286]). In turn, the high levels of Wg and Cut expression within the D/V boundery specify the wing margin by the end of larval development [287].

The Drosophila wing margin is composed of hair and innervated sensory bristles. The sensory bristles found on the anterior margin, are divided in three classes: chemosensory, slender and stout mechanosensory bristles [288]. These bristles are ordered on the wing margin in a precise pattern, which will be described in section 3.1.1. The sensory organs do not differentiate before pupariation, after the specification of the wing margin. First, proneural proteins Achaete, Scute, Daughterless and Senseless promote the formation and the survival of many sensory organ precursors (SOPs) in the wing disc epithelium [289]. Within the prospective wing margin, the SOP differentiates into at least four cell-types: the neuron, and the sheath, shaft, and bristle cells [288]. The differentiation of SOPs is regulated by asymmetric cell division which dictates the position and the fate of each cell type (reviewed in [290, 291]). For instance, cell fate is primarily defined by the uneven distribution of the Notch signalling inhibitor, Numb, between newly divided daughter cells [291]. In light of the specificity of each signalling pathway that regulates its patterning, the *Drosophila* wing can be used as a model in which to study gene function in a selected developmental process.

1.5 Project

During the course this research project, the function of *Drosophila* PDZ-GEF was studied within the wing margin by various methods. The background and rationale of the project are explained in the following sections.

1.5.1 Background

A modifier screen was performed in our laboratory in order to identify novel members and collaborators of the Ras-MAPK pathway in *Drosophila*. The strategy was to screen for mutant alleles that modified the rough eye phenotype induced by the dominant negative effect of C-terminal CNK on Ras-MAPK signalling (see section 1.2.3). Firstly,

males that had been mutagenized with EMS were crossed with transgenic females expressing cnk^{CT} under the control of the eye-specific Sevenless enhancer (sE) [292]. Secondly, the progeny was screened for individuals that had an enhanced rough eye phenotype; an indication that they may carry a mutation within a locus that genetically interacts with *cnk* or members of a pathway in which CNK is involved. Finally, many of the alleles that were isolated have turned out to be a mutated version of known components of the Ras-MAPK pathway such as EGFR, Ras and MAPK. However, two groups of alleles were mapped and identified as the small GTPase Rap1 and its activator PDZ-GEF. Upon closer examination, Rap1 and PDZ-GEF alleles recovered from the screen not only enhanced the rough eye phenotype induced by the expression on cnk^{CT}, but they increased the number of missing photoreceptor cells within ommatidia. Moreover, in combination with cnk^{CT}, these mutant alleles caused ommatidia to appear misshapen and fused together, suggesting that Rap1 and dPDZ-GEF may regulate photoreceptor differentiation and eye morphology via a functional interaction with CNK (Figure 2A). Consequently, the recovery of alleles for both Rap1 and dPDZ-GEF led our laboratory to study these proteins in *Drosophila*, in order to further characterize the PDZ-GEF/Rap1 pathway and eventually define a functional relationship between PDZ-GEF/Rap1 signalling and CNK, or the Ras-MAPK pathway.

Six mutant alleles of dPDZ-GEF were isolated from the screen, and five were molecularly characterized. One allele carries a point mutation within the cNMP domain (E-174). There are four alleles that carry mutations of conserved residues within the GEF domain: three alleles carry a point mutation (E-185, E-322, E-323) and one carries a deletion of four amino acids (E-696) (Figure 2B). Flies homozygous for these alleles, except E-696 which is recessive lethal, have a rough eye and exhibit wing defects. For instance, the sensory bristles found on the wing margin are irregularly spaced, thus forming clumps of bristles and gaps along the margin (Figure 2). Interestingly, the number of wing margin bristles was the same as in wild-type animals.

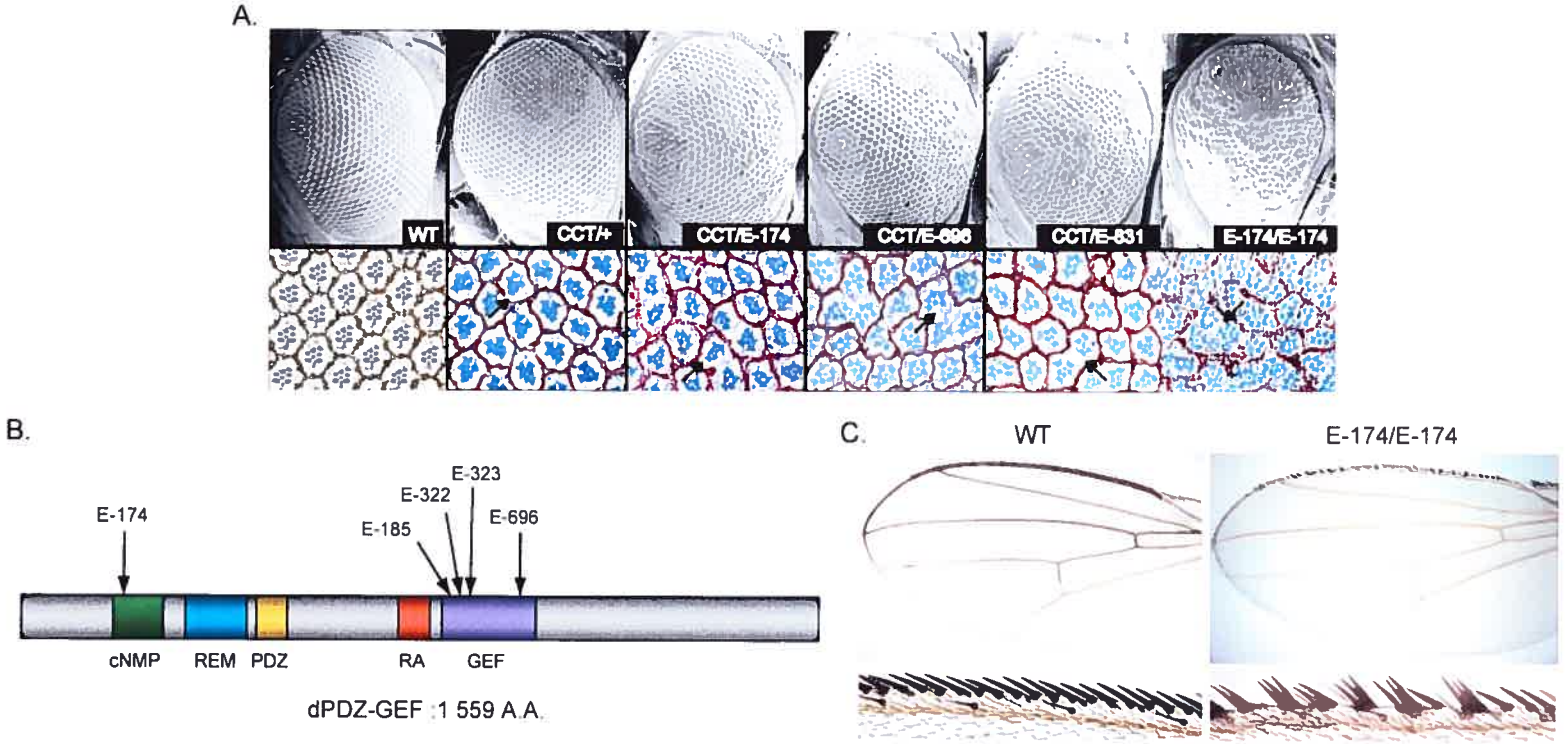

Figure 2. Alleles of *dRap* and *dPDZ-GEF* were isolated from the CNK^{CT} screen (M. Lefrançois and M. Therrien, unpublished)
(A) Scanning electron microscopy and tangential sections of adult *Drosophila* eyes. *dPDZ-GEF* alleles E-174 and E-696, and *dRap* allele E-831 enhance the CNK^{CT} (CCT) rough eye phenotype. The rough eye phenotype is characterized by the loss of photoreceptor cells and some fused ommatidia (black arrows). Homozygous *dPDZ-GEF*$^{E-174/E-174}$ mutants also exhibit a rough eye phenotype, missing photoreceptors and fused ommatidia. (B) Graphical representation of the domains and characterized alleles of dPDZ-GEF (E-174: D to V; E-185: M to I; E-322: C to Y; E-323: R to C; E-696: 4 A.A. deletion (IHLG). (C) In *dPDZ-GEF*$^{E-174/E-174}$ mutants, sensory bristles are abnormally spaced along the anterior wing margin when compared to wild-type (WT). cNMP: cyclic nucleotide monophosphate binding domain; REM: Ras-exchange motif; PDZ: PSD-95/Dlg/ZO-1; RA: Ras/Rap1 associating; GEF: catalytic domain.

Figure 2: Description of the CNK^{CT} screen

1.5.2 Overview

The main objective of this research project was to study the function of dPDZ-GEF. To do so, the wing margin phenotype induced by the alleles recovered from the screen was characterized at macroscopic and microscopic levels. In order to identify the cellular processes in which PDZ-GEF might be involved, immunohistochemistry of *Drosophila* wild-type and mutant wing discs was performed. This method has not only provided a better understanding of the morphology of the anterior wing margin during pupal development, but has offered intriguing insight on the function of dPDZ-GEF. Moreover, the interaction between dPDZ-GEF with CNK was genetically assessed and confirmed within the wing margin. Finally, preliminary characterization of tools to aid in the study of dPDZ-GEF in the future was also performed. These results as well as the hypotheses as to their significance are described in the following sections.

CHAPTER 2 : MATERIALS AND METHODS

2.1 *Drosophila* stocks

Isogenic line W^{iso} was used as wild-type. *dPDZ-GEF* allles (E-322, E-174, E-696) were isolated from the CNK^{CT} genetic screen performed in the laboratory by M. Lefrançois. Homozygous $dPDZ\text{-}GEF^{mutant}$ flies were obtained by mating males with virgin females of the same genotype. Transheterozygous $dPDZ\text{-}GEF^{mutant}$ flies were obtained by mating males of one genotype and virgin females of another. $dPDZ\text{-}GEF^{E\text{-}174}/CyO;C96\text{-}Gal4/TM3$, $dPDZ\text{-}GEF^{E\text{-}322}/CyO;C96\text{-}Gal4/TM3$, $dPDZ\text{-}GEF^{E\text{-}696}/CyO;C96\text{-}Gal4/TM3$, $dPDZ\text{-}GEF^{P13720}/CyO;C96\text{-}Gal4/TM3$, and *E-174-FRT/CyO-TM6B* were generated by M. Lefrançois. *hs-FLP; E-174-FRT/Cyo-TM6b* was generated from hs-FLP (G.M. Rubin) and *174-FRT/CyO-TM6B*. Null allele $dPDZ\text{-}GEF^{P13720}$ and $Ubi\text{-}GFP\text{-}FRT^{40A}$ were obtained from the Bloomington stock center. $UAS\text{-}CNK^{CT}$ was generated by M. Lefrançois and *C96-Gal4/CyO-TM6B* was obtained from G.L. Boulianne. All fly stocks were raised at 25C°.

2.2 Generation of mitotic clones

Transgenic lines expressing yeast Flp recombinase and FRT (Flp recombinase target) sites are used to generate mitotic clones in *Drosophila*. The recombinase mediates site-specific recombination between homologous chromosomes that carry transgenic FRT sites inserted at the same genomic location [293]. Following replication, chromatids that carry homologous portions of a chromosome can segregate together and generate a daughter cell homozygous for that genomic region. This method is widely used to generate mitotic clones homozygous for a mutant allele within an otherwise wild-type animal [294]. Moreover, mitotic clones can be generated in specific tissues by placing the FLP recombinase transgene under the control of a tissue-specific regulatory element. Consequently, the FLP-FRT system can be used to study the phenotypes induced by alleles that are recessive lethal, or to compare mutant and wild-type tissues in the same organism. For the purpose of our study $Ubi\text{-}GFP\text{-}FRT^{40A}$ males were mated with virgin females of genotype *hs-FLP; E-174-FRT/Cyo-TM6b*. Embryos were heat-shocked at 37°C for 45 minutes at 36 hours after egg laying (AEL), 60h AEL and 74h AEL. Prepupae were subsequently collected for antibody staining.

2.3 Immunofluorescence and microscopy

Larval wing discs were collected at the third instar of larval development. Prepupae were staged at approximately day 6, and pupal wing discs were collected 28h after pupal formation (APF). Larval and pupal wing discs were dissected in PBS and fixed for 15 minutes in 4% paraformaldehyde diluted in PBS. After three washes in PBT (PBS and 0.2% Triton X-100), the wing discs were blocked for 15 minutes in PBT containing 2% BSA (unless stated otherwise), and incubated overnight at 4°C with primary antibodies in PBT containing 2% BSA. After three washes in PBT, the wing discs were incubated for 2 hours at room temperature with secondary antibodies (also DAPI or Phalloidin-TRITC in some cases) in PBT containing 2% BSA. After three more washes in PBT, wing discs were mounted in Vectashield mounting medium (Vector Laboratories). The images of the antibody staining of larval wing discs were obtained with a Zeiss Axio Imager Z1 upright microsope with the Axio Vision 3.2 acquisition system. Confocal microscopy images were obtained with a Zeiss LSM510 system. All images were processed using Adobe Photoshop CS.

The following antibodies were used: anti-Wingless (1:10 blocked in 5% milk diluted in PBT and incubated in 2.5% milk; Developmental Studies Hybridoma Bank (DSHB)), anti-Cut (1:200; DSHB), anti-Scabrous (1:200; DSHB), anti-Elav (1:1000; G.M. Rubin), 22C10 (anti-neurons) (1:50; DSHB), anti-Dlg (1:1000; DSHB), anti-Fasciclin III (1:10; DSHB), anti-Armadillo (1:50; DSHB), anti-dE-Cadherin (1:10; DSHB), anti-βPS (1:50 or 1:20 for mitotic clones; DSHB), anti-αPS1 (1:20; DSHB), purified and concentrated pre-immmune bleed anti dPDZ-GEF (1:20; generated by Sigma Genosys). Secondary antibodies were anti-mouse anti-rat IgG conjugated to Cy3 or anti-rat Cy5 respectively (1:1000; Jackson ImmunoResearch), and anti-mouse or anti-rabbit IgG conjugated to Alexa 488 or Alexa 555 (1:1000; Invitrogen). DAPI was used to stain DNA, and Phalloidin-TRITC (Sigma) was used to stain filamentous actin.

2.4 Analysis of adult wings and genetic interaction between *dPDZ-GEF* alleles and *cnk*^{CT}

Adult wings were dissected in 70% EtOH, and rinced in water. The rinsed wings were mounted in Canada Basalm (Sigma), and images were taken using a Leica MZFLIII stereomicroscope with the Northern Eclipse acquisition system (EMPIX Imaging Inc.).

W^{iso}, *dPDZ-GEF*$^{E-174}$*/CyO;C96-Gal4/TM3*, *dPDZ-GEF*$^{E-322}$*/CyO;C96-Gal4/TM3*, *dPDZ-GEF*$^{E-696}$*/CyO;C96-Gal4/TM3* and *dPDZ-GEF*^{P13720}*/CyO;C96-Gal4/TM3* males were mated with *UAS-CNK*^{CT} virgin females. +/+;*UAS-CNK*^{CT}*/C96-Gal4* and *dPDZ-GEF*^{mutant}*/+; UAS-CNK*^{CT}*/C96-Gal4* males and females were analysed. The percentage of flies with wing defects was calculated from the total number of flies counted. The experiment was repeated twice. In the experiment represented, the total amount of flies counted for each genotype is: *UAS-CNK*^{CT}*/C96-Gal4* (55), *E-174* (38), *E-322* (164), *E-696* (58), *P13720* (75).

2.5 Plasmids

The GST-dPDZ-GEF^{CT} plasmid (M. Lefrançois) was contructed by inserting a PCR product corresponding to a non-conserved region of dPDZ-GEF and generated with OL5'dPDZ-GEF (GTGGAATTCTGGAATGCGAGCCCGC GCAC) and OL3'dPDZ-GEF (GTGCTCGAGAAGCTTTTATTGATTCATGGGTGG CATGGG) into the EcoRI/XhoI restriction sites of pGEX-4T-2 (GE Healthcare). pMAL-dPDZ-GEF^{CT} (M. Therrien) was constructed by inserting a PCR product corresponding to a non-conserved region of dPDZ-GEF and generated with OL5'dPDZ-GEF (GTCGAATTCCTGGAATGCGAGCCCGCGC ACGGG) and OL3'dPDZ-GEF (GTCAAGCTTTTATTGATTCATGGGTGGCATGGG AC) into the EcoR1/HindIII restriction sites of pMAL (New England Biolabs). pWIZ-PDZ-GEF (M. Therrien) was constructed by inserting a PCR product corresponding to a non-conserved region of dPDZ-GEF and generated with OL5'dPDZ-GEF (GTCTCCTAGGGTCTTACCGTGCGGGATGCCGTGCGTG) and OL3'dPDZ-GEF (GTCTGCTAGCGGTTCTGAATAAAGATGCGGTGTG) into the AVRII/NHEI restriction sites of pWIZ (Drosophila Genomics Resource Center). FLAG-dPDZ-GEF (M.

Lefrançois) was generated by subcloning a DNA fragment corresponding to dPDZ-GEF into the XmaI/NotI restriction sites of pMet/FLAG (a vector containing the *metallothionein* promoter that is inducible by heavy metals and the FLAG epitope). FLAG-dPDZ-GEFΔcNMP (M. Lefrançois) was generated by subcloning a DNA fragment corresponding to dPDZ-GEFΔcNMP (starting at amino acid 234) into the XmaI/NotI restriction sites of pMet/FLAG. pMet-Gal4 was obtained from G. Laberge.

2.6 Generation and purification of anti-dPDZ-GEF

IPTG (100 mM) was used to induce the expression of GST-dPDZ-GEF^{CT} in *E. coli* BL21 competent cells at 37°C. Following centrifugation at 3,000 rpm for 20 min (4°C), the bacterial pellet was resuspended in phosphate-BME buffer (PMSF 100mM, EDTA 500mM, β-mercaptoethanol 10mM diluted in PBS) and lysed with a lysosyme solution (50 mg/mL). The lysate was freezed in an EtOH/CO_2bath, thawed and sonicated three times for 15 seconds. 1% Triton X-100 was added and centrifuged at 3,000 rpm (4°C). Following centrifugation, the lysate was incubated with Glutathione Sepharose 4B (Amersham Biosciences) for 2 hours at room temperature. The resin was washed three times with PBS, and the eluate was collected after 15 minutes of incubation with 5mM glutathione diluted in Tris-HCl 1M pH8. The protein extract was dialysed against PBS, and sent to Sigma Genosys for production of the antibody in two rabbits. The antisera from one of these rabbits were used for this work. All concentrations represent final concentrations, unless otherwise stated.

Anti-dPDZ-GEF (third bleed) and the pre-immune bleed were purified using a MBP-dPDZ-GEF^{CT} affinity column. IPTG (100 mM) was used to induce the expression of pMAL-dPDZ-GEF^{CT} in *E. coli* BL-21 competent cells at 30°C. Following centrifugation at 3,000 rpm for 20 min (4°C), the bacterial pellet was resuspended in MBP buffer (EDTA 200mM diluted in PBS) with inhibitors (β-mercaptoethanol 10mM, PMSF 100mM, aprotinin 0.15U/mL, leupeptin 20 μM) and lysed with a lysosyme solution (50 mg/mL). The lysate was freezed in an EtOH/CO_2bath, thawed and sonicated six times for 15 seconds. Following centrifugation at 4°C, the lysate was incubated with an amylose resin (New England BioLabs) for 2 hours at room temperature. The resin was washed several

times with NaCl 0.5M buffers: a carbonate coupling buffer (NaHCO3 0.1M pH 8.3), an acetate buffer (NaHCO3 0.1M pH 4.5), and a storage buffer (NaN3 0.2%). The resin was then transferred to a column. Following additional washes with glycine-HCl 0.1 M pH 2.8 and PBS, 1 mL of anti-dPDZ-GEF or pre-immune bleed was applied to the column. The column was washed three times with PBS, and the eluate (10 fractions) was collected with a final glycine-HCl pH 2.8 wash. The fractions were neutralized with 1M Tris base pH 7.8 to a volume of approximately 1/5 of the total volume of the fraction. For the concentration of anti-dPDZ-GEF (third bleed) and the pre-immune bleed, the fractions were pooled and centrifugated at 3,000 rpm for 45 min using an Ultracel YM 10MW cellulose membrane Centricon centrifugal filter device (Amicon). Concentrated products of approximately 250 µL each were obtained. All concentrations represent final concentrations, unless otherwise stated.

2.7 Cell culture, transfection, extracts and protein immunoprecipitation

S2 cells were maintained in FBS-containing Schneider medium (Invitrogen). For transfection experiments, 8 X 10^6 (or 20 X10^6) cells were plated per 60-mm (or 100-mm) dish and transfected the next day with various plasmid combinations using the Effectene transfection reagent (Qiagen). $CuSO_4$ (0.7 mM) was added to the medium 24h after transfection to induce protein expression.

Cells were harvested and centrifuged at 1,000 rpm for 5 min (4°C) 24 hours after induction. The pellet was resuspended and lysed in NP-40 lysis buffer (20mM Tris-HCl pH 8, 1% NP-40, 137mM NaCl, 10% Glycerol, 1mM EDTA) with inhibitors (PMSF 0.5mM, aprotinin 0.15U/mL, leupeptin 20 µM). The lysates were collected after centrifiguation of cell debris at 14,000 rpm (4°C).

For immunoprecipitations, cell lysates (3mg total proteins) were incubated with anti-FLAG (Sigma) and protein A/G PLUS-Agarose beads (Santa Cruz Biotechnology, Inc.) for 4 hours at 4°C. Immunoprecipitated proteins were then washed three times with cold lysis buffer before analysis.

2.8 Protein analysis

Total cell lysates (100 μg of protein) or immunoprecipitates from 3 mg of cellular extracts were resolved on 8% SDS-PAGE. Coomassie blue staining was performed by incubating the SDS-PAGE with Coomassie Blue stain for 1 hour and destaining (10% acetic acid and 25% EtOH) for at least 3 hours. For western blots, proteins resolved on SDS-PAGE were transferred to nitrocellulose membranes. BSA (New England BioLabs) was used as a control.Anti-FLAG (1:20 000), anti-Src42A (1:1000; J. Dickson), anti-dPDZ-GEF [first, second and third bleed; third bleed after purification and third bleed after concentration] (1:100) and the pre-immune bleed (1:100) were used as primary antibodies. Secondary antibodies were anti-mouse (Calbiochem) and anti-rabbit (Santa Cruz Biotechnology, Inc) IgG-HRP (1:20 000 and 1:1 0000 respectively).

CHAPTER 3 : RESULTS

3.1 Characterization of the function of dPDZ-GEF in the *Drosophila* anterior wing margin

The main objective of this research project was to characterize the wing margin phenotype induced by the alleles recovered from the screen (Figure 2A). Consequently, the anterior wing margin of both wild-type and mutant flies was characterized at the macroscopic and microscopic level.

3.1.1 *dPDZ-GEF* mutant alleles induce defects in the spacing of mechanosensory bristles in the adult anterior wing margin

In wild-type adult flies, the sensory bristles are stereotypically spaced along the anterior wing margin. The stout mechanosensory bristles are centrally located at the edge of the wing margin, slender mechanosensory bristles are on the ventral side, and chemosensory bristles are found on both the dorsal and ventral side of the wing margin (Figure 3). Wing margin bristles appear to be irregularly spaced in flies homozygous for mutant alleles *dPDZ-GEF* $^{E\text{-}174}$ or $^{E\text{-}322}$, though the phenotype induced by the expression of E-322 is weaker (Figure 3A and data not shown). Upon closer examination, the number of bristles is the same as in wild-type (not shown), and the spacing defect seems to affect stout and slender mechanosensory bristles, but not chemosensory bristles (Figure 3B). Homozygous *dPDZ-GEF*$^{E\text{-}174}$ flies occasionally present altered longitudinal spacing of their mechanosensory bristles which creates tufts of closely grouped bristles separated by large gaps along the wing margin (Figure 3B black arrows). The recessive lethal allele *dPDZ-GEF*$^{E\text{-}696}$ induces an enhanced phenotype when it is combined with *dPDZ-GEF* $^{E\text{-}174}$, while *dPDZ-GEF*$^{E\text{-}322}$, which is homozygous viable, reduces the intensity of the phenotype. These results suggest that *dPDZ-GEF* alleles alter the spacing of mechanosensory bristles along the adult wing margin. Nevertheless, the polarity or the number of the bristles on the wing margin or wing blade does not seem to be affected by the mutant alleles, suggesting that dPDZ-GEF is not involved in the determination of planar polarity or sensory organ number in the wing epithelium.

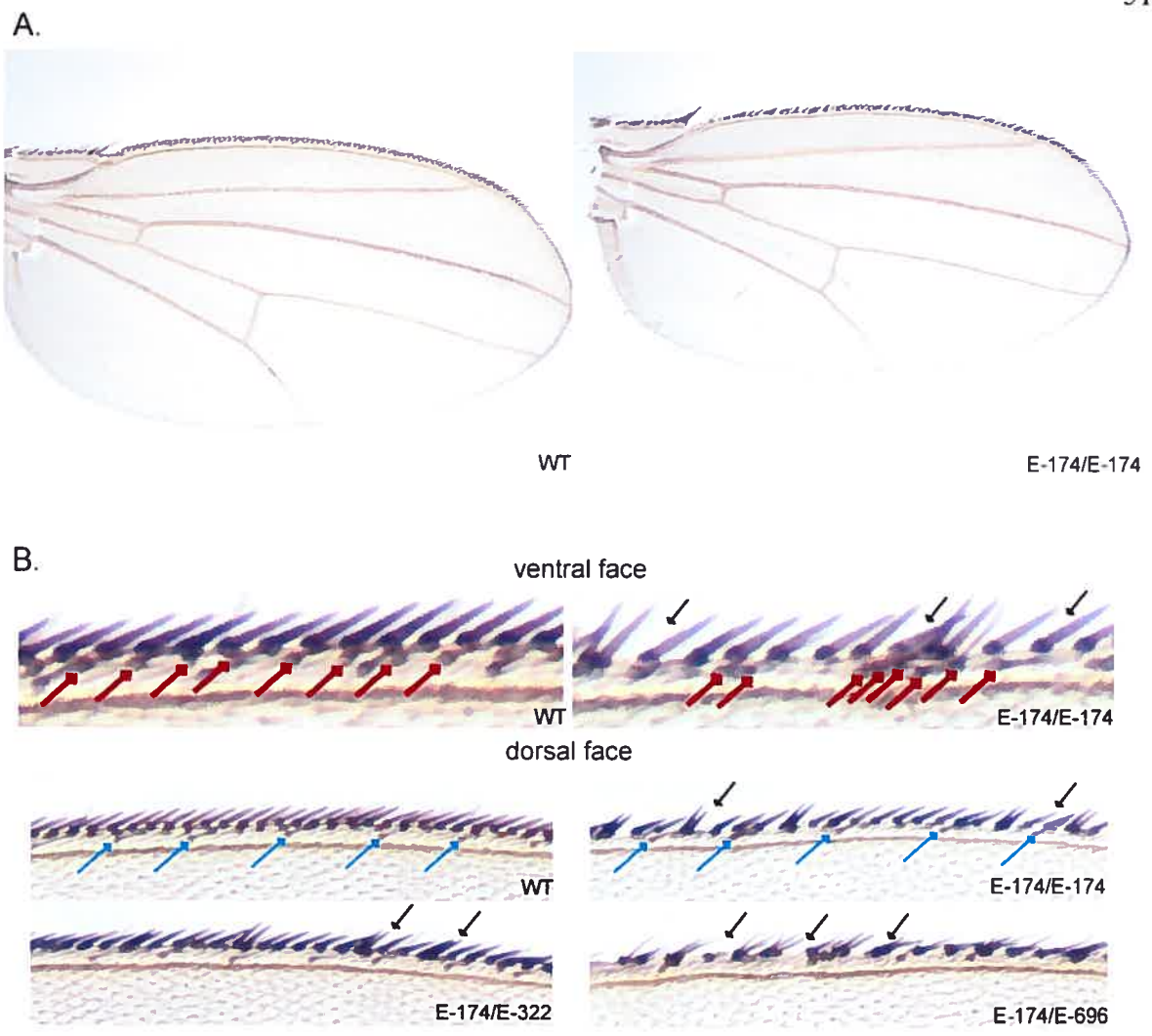

Figure 3. dPDZ-GEF alleles disrupt the spacing of mechanosensory bristles in the adult wing margin. (A-B) Adult *Drosophila* wings of the following genotypes: wild-type (WT), homozygous *dPDZ-GEF*$^{E\text{-}174/E\text{-}174}$, and transheterozygous *dPDZ-GEF*$^{E\text{-}174/E\text{-}322\ or\ E\text{-}174/E\text{-}322}$. (A) In *dPDZ-GEF*$^{E\text{-}174/E\text{-}174}$ mutants, sensory bristles are abnormally spaced along the anterior wing margin (AWM). (B) In *dPDZ-GEF*$^{E\text{-}174/E\text{-}174}$ mutants, a close-up of the AWM reveals that slender mechanosensory bristles are unevenly spaced when an alignment of 8 bristles is compared to WT (red arrows on the ventral face). Stout mechanosensory bristles are also unevenly spaced (black arrows) in mutants, but chemosensory bristles are not affected (blue arrows). When compared to *dPDZ-GEF*$^{E\text{-}174/E\text{-}174}$, *dPDZ-GEF*$^{E\text{-}174/E\text{-}322}$ has a weaker phenotype, and *dPDZ-GEF*$^{E\text{-}174/E\text{-}696}$ an enhanced phenotype.

Figure 3: Phenotypic effects of *dPDZ-GEF* alleles in the wing margin

Moreover, the genetic interaction between the *dPDZ-GEF* alleles demonstrates that they form an allelic series of mutations with E-322 inducing the weakest phenotype, E-696 inducing the strongest, and E-174 being of midrange strength. Interestingly, when *dPDZ-GEF* alleles, including the null allele *dPDZ-GEF*^{P13720}, are expressed in combination with a deficiency of the locus, they produce various intensities of the spacing defect described above (M. Lefrançois, personal communication). Thus, these results suggest that the alleles recovered from the CNK^{CT} screen may be loss of function mutations. Since *dPDZ-*$^{GEFE-174}$ produces the strongest phenotype among homozygous viable alleles, we have predominantly used this allele to induce a mutant phenotype in subsequent analyses.

3.1.2 *dPDZ-GEF* mutant alleles do not affect wing margin development in larvae

Following the characterization of the external phenotype in the adult fly, we wanted to characterize the phenotype at the cellular level. Since the adult wing is a cuticular structure, the internal effects of *dPDZ-GEF* alleles were assessed by immunohistochemistry of developing wing discs. In the third instar of larval development, the prospective wing margin is specified by the expression of proteins that regulate patterning of the epithelium. Thus, I assessed whether wing margin determinants were aberrantly expressed in L3 larvae. The proteins Wingless and Cut were selected for antibody staining as they regulate wing margin patterning during larval development (refer to section 1.4.1.2.2). As described in Figure 4A, Wg and Ct expression is restricted to a three-cell wide stripe that runs across the D/V boundery of the prospective wing margin epithelium. In larvae homozygous for *dPDZ-GEF*$^{E-174}$, the expression pattern of both Wg and Cut appears to be comparable to wild-type (Figure 4A).

Since early wing margin determinants appear to be unaffected by *dPDZ-GEF* alleles, I next wanted to verify if sensory organ precursor (SOP) differentiation and distribution along the prospective anterior wing margin was impaired in mutants. The neurogenic protein Scabrous (Sca), a target of the proneural achaete-scute complex and a potential antagonist of Notch signalling in the wing margin, was selected as a marker of SOP differentiation [295, 296]. Within the prospective wing margin, the expression of Sca

Figure 4. Wingless, Cut and Scabrous expression is not affected by *dPDZ-GEF* alleles.
(A-B) Immunofluorescence images of *Drosophila* wing discs at the third larval instar for the following genotypes: wild-type (WT), homozygous *dPDZ-GEF*$^{E\text{-}174/E\text{-}174}$, transheterozygous *dPDZ-GEF*$^{E\text{-}174/E\text{-}696}$ and hs-FLP; E-174,FRT^{40A}/ubi-GFP,FRT^{40A}. (A) Wingless (Wg) and Cut are expressed in the prospective wing margin along the D/V boundary (long arrow), and the expression pattern of *dPDZ-GEF*$^{E\text{-}174/E\text{-}174}$ mutants appears to be the same as wild-type. (B) In mosaic individuals, wing margin expression of Scabrous (Sca) (short arrow) appears to be the same in WT (GFP) and *dPDZ-GEF*$^{E\text{-}174}$ mutant clones. *dPDZ-GEF*$^{E\text{-}174/E\text{-}696}$ mutants also appear to have normal Sca expression. The intensity of the Sca staining varies according to the time at which the wing discs were collected (mosaics:late third instar, *dPDZ-GEF*$^{E\text{-}174/E\text{-}696}$ early third instar).

Figure 4: Larval characterization of the wing margin in *dPDZ-GEF* mutants

is restricted to the anterior compartment within two distinct cell stripes in the D/V boundery (Figure 4B). Mitotic clones of the *dPDZ-GEF*$^{E-174}$ allele were generated under the control of an inducible heat-shock protein promoter, and Sca expression is seemingly unaffected in mutant tissue when compared to wild-type tissue. Moreover, in transheterozygous *dPDZ-GEF*$^{E-174/E-696}$ mutants, the expression of Sca appears to be undisturbed. Conclusively, these results suggest that dPDZ-GEF may not be involved in early wing margin patterning. However, this hypothesis remains to be proven.

3.1.3 *dPDZ-GEF* mutant alleles disrupt the morphology of sensory organs during pupal wing margin development

Since dPDZ-GEF is seemingly not involved in early wing margin development, later stages of development of wing development were investigated. As mentioned in the introduction, SOPs initiate their differentiation during pupal development, thus the pupal wing disc was selected to pursue our study. During pupariation, the wing disc undergoes several modifications including differentiation and morphogenetic movements.In order to define the complex morphology of the anterior wing margin, we performed immunohistochemistry on pupal wing discs and analysed by confocal microscopy. Antibody staining of the neuron-specific RNA-binding protein Elav was used to monitor the development of sensory neurons along the prospective anterior wing margin, and the anti-neurons/22C10 antibody was used to visualize axons (Figure 5). At 28 hours after pupal formation (APF), all the neurons are specified and exhibit a bipolar structure (Figure 5A). The mechanosensory bristles are innervated by one bipolar neuron, and the chemosensory bristles are innervated by five (Figure 5A, WT). Neurons extend one axon to the edge of the wing disc where the external structure of the bristle will form, while the other axon extends into a fascia which groups all the sensory axons into a tight bundle located at what appears to be the prospective L_1 vein (Figure 5A). In wild-type pupae, dorsal and ventral sensory neurons are aligned and regularly spaced on top of the axonal fascia (Figure 5A, WT). However, the sensory neurons of pupae homozygous for *dPDZ-GEF*$^{E-174}$ are unevenly spaced and appear detached from the axonal fascia (Figure 5A, E-174/E-174). Moreover, the axons within the fascia do not seem to be as tightly bound

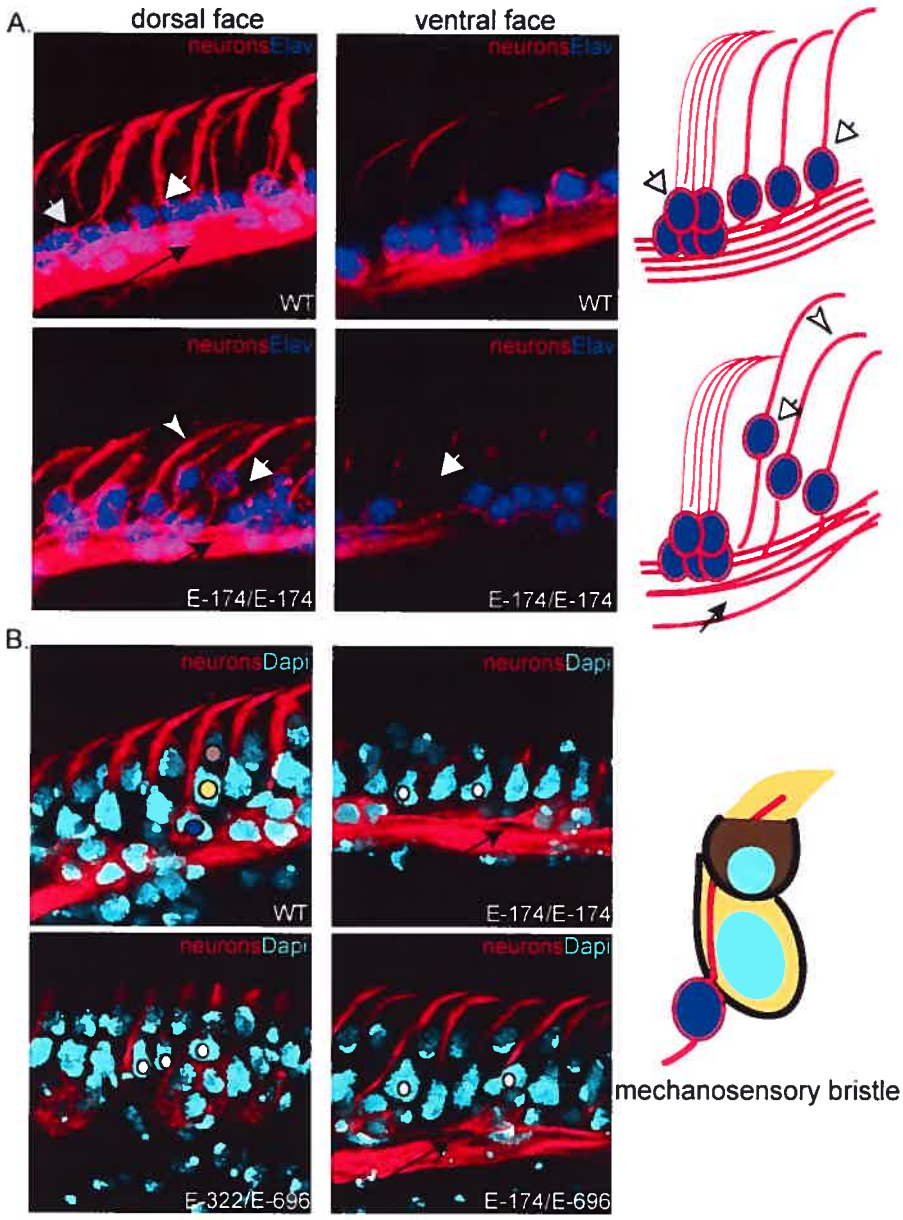

Figure 5. *dPDZ-GEF* alleles disrupt the morphology of the anterior wing margin in pupae. (A-B) Images of the anterior wing margin of pupae at 28h APF taken by confocal microscopy for the following genotypes: wild-type (WT), homozygous *dPDZ-GEF*$^{E-174/E-174}$, transheterozygous *dPDZ-GEF*$^{E-322/E-696}$. (A) In WT pupae, mechanosensory amd chemosensory neurons (anti-Elav; white and grey short arrows respectively) are aligned evenly on the axonal fascia (anti-neurons/22C10). In mutant pupae, neurons appear detached from the axonal fascia and unevenly spaced, while axons exhibit impaired adhesion (black arrows) and orientation (white arrowheads). The dorsal views are 3D reconstructions of confocal planes taken at intervals of 0.8 µm. (B) Homozygous and transheterozygous *dPDZ-GEF* mutants have deformed nuclei (white dots). From top to bottom, the drawings represent the WT and mutant anterionr wing margin, and cells within a mechanosensory bristle: socket cell (brown), bristle cell (yellow) and neuron (blue). All images were acquired with a 100X objective lens and zoomed 2 fold.

Figure 5: Characterization of the anterior wing margin in *dPDZ-GEF* mutants

together when compared to wild-type (Figure 5A), and the orientation of axons at the edge of the wing disc appears to be impaired. The same observations can be made in transheterozygous *dPDZ-GEF*$^{E\text{-}174/E\text{-}696}$ and *dPDZ-GEF*$^{E\text{-}322/E\text{-}696}$ pupae (Figure 5B). Finally, we looked at the overall morphology of the prospective anterior wing margin, where each sensory organ is composed of at least four cell-types: a neuron (blue dot), a shaft/hair cell (trichogen; yellow dot), a socket cell (tormogen, brown dot) and a sheath cell (techogen, out of focal plane) (Figure 5B, WT). The sensory organs of homozygous and transheterozygous *dPDZ-GEF*^{mutant} pupae exhibit aberrant cell shapes when they are compared to wild-type, as hair cell nuclei appear deformed and misaligned (Figure 5B). Additionally, the nuclei of hair, socket and neuronal cells cannot be visualized at the same focal plane, suggesting a disorganization of the cells within the sensory bristles of these mutants (Figure 5B). Interestingly, the phenotypes induced by *dPDZ-GEF* alleles appear to involve improper cell adhesion and cell morphology. Altogether, these results suggest that in the anterior wing margin of pupae (28h APF) dPDZ-GEF activity might dictate the localization and the morphology of sensory organs.

3.1.4 dPDZ-GEF may regulate the localization of adherens junction proteins in the pupal anterior wing margin

Since neurons are detached from the axonal fascia and axons appear delaminated within the fascia *dPDZ-GEF* mutant pupae, cell adhesion within wild-type and mutant sensory organs was assessed by antibody staining of known adhesion molecules found at cellular junctions. Since septate junctions are known to mediate axon-cell and axon-axon adhesion, the expression pattern of septate junction proteins Fasciclin III and Dlg was assessed [297]. At 28h APF, Dlg and Fas III are expressed at the membrane of neurons, socket and hair cells, as well as within the axonal fascia (Figures 6 and 7). However, Dlg is predominantly expressed in socket cell membranes at the most apical region of the anterior wing margin (Figure 6A). The expression of Dlg and Fas III is comparable to wild-type in pupae homozygous for *dPDZ-GEF*$^{E\text{-}174}$ (compare A and B to C and D in Figures 6 and 7). Moreover, we have induced mitotic clones of *dPDZ-GEF*$^{E\text{-}174}$, and mosaic pupae appear to have normal Dlg and Fas III expression along the anterior wing margin in wild-type (GFP)

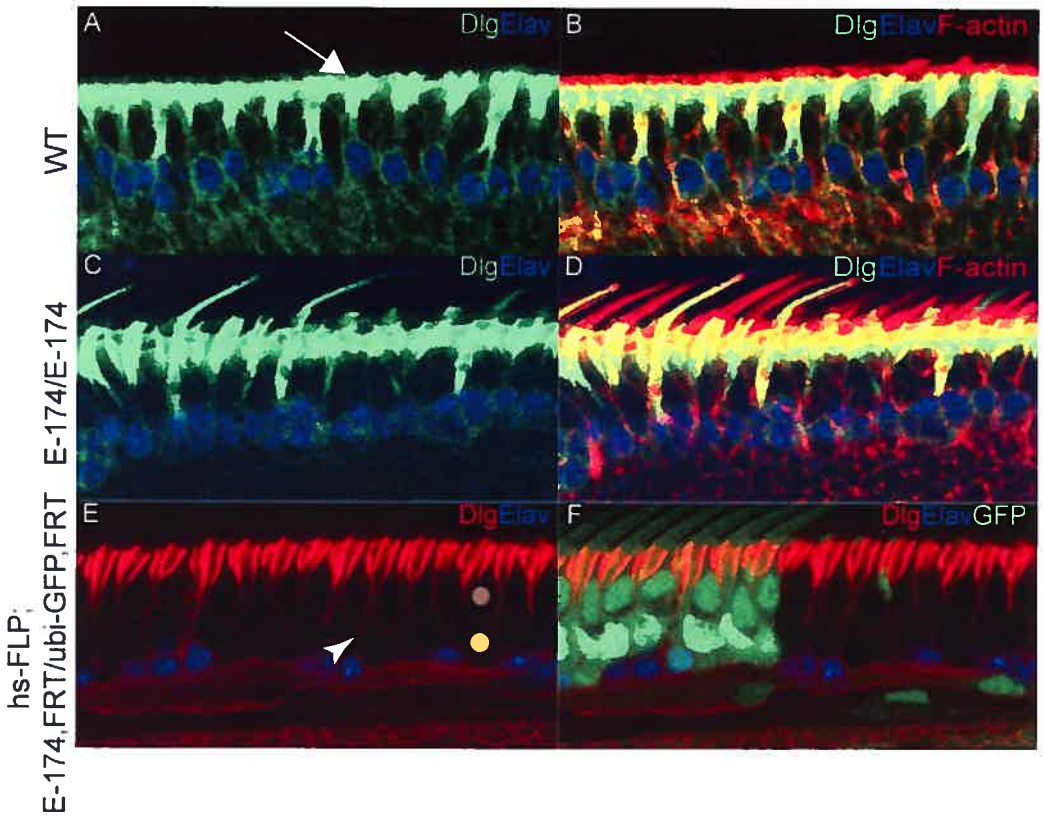

Figure 6. A *dPDZ-GEF* mutant allele has no effect on the localization of Dlg.
(A-F) Images of the anterior wing margin of pupae at 28h APF for the following genotypes: wild-type (WT): homozygous *dPDZ-GEF*$^{E-174/E-174}$; hs-FLP; *dPDZ-GEF*$^{E-174}$,FRT^{40A}/ubi-GFP,FRT^{40A}. (A,B) Dlg is predominantly expressed at the membrane of socket cells at the edge of the wing margin (white arrow) and partly colocalizes with F-actin. (C,D) The localization of Dlg is unaffected by *dPDZ-GEF*$^{E-174}$. (E,F). Socket cells (brown dot) are abnormally elongated in mutant *dPDZ-GEF*$^{E-174}$ clones (arrowhead) compared to wild-type clones (GFP). All images were acquired by confocal microscopy with a 63X objective lens and zoomed 2 fold. (the yellow dot represents hair cells and neurons are labelled with anti-Elav)

Figure 6: Characterization of septate junctions in *dPDZ-GEF* mutants (I)

and mutant tissues (Figure 6; E and F). Nonetheless, labelling with anti-Dlg or anti-Fas III reveals elongated socket cells in homozygous *dPDZ-GEF*$^{E-174/E-174}$ clones, as well as pupae homozygous for *dPDZ-GEF*$^{E-174}$ (Figure 6E, Figure 7 C and E). Though we have acquired further confirmation that *dPDZ-GEF* mutations alter the morphology of cells at the anterior wing margin, *dPDZ-GEF* alleles do not seem to alter the localization of septate junction proteins Fasciclin and Dlg.

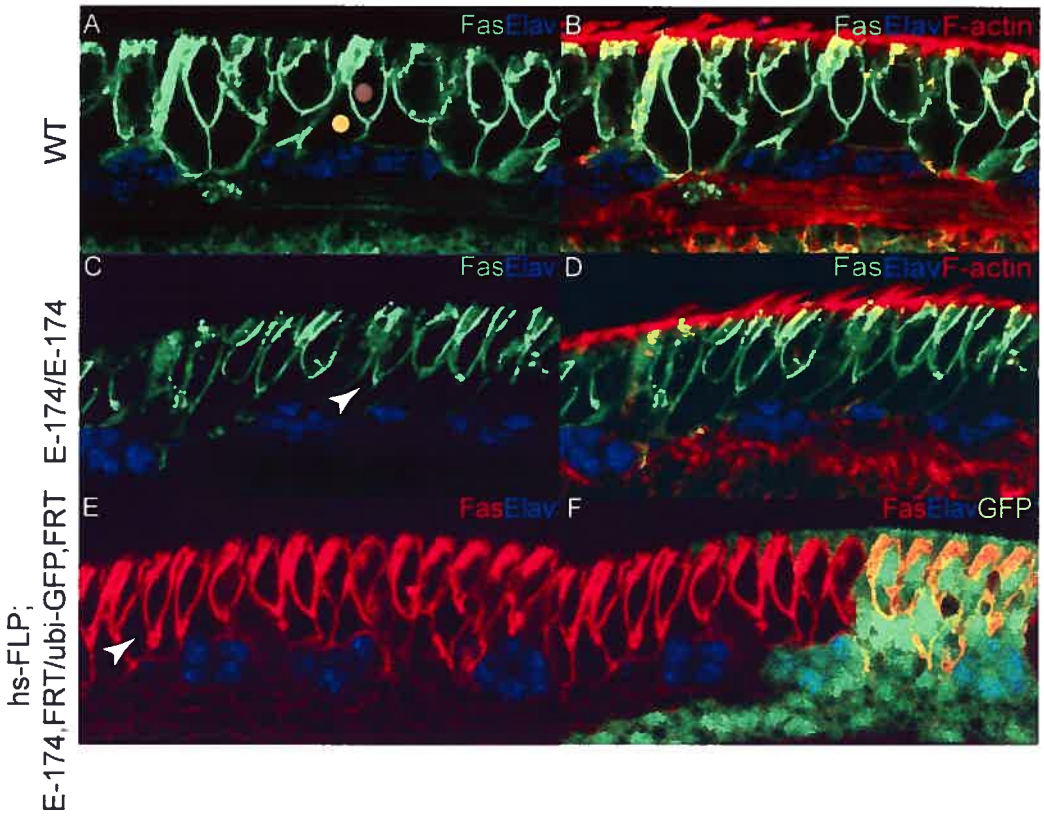

Figure 7. The expression of *dPDZ-GEF* alleles has no effect of septate junctions. (A-F) Images of the anterior wing margin of pupae at 28h APF for the following genotypes: wild-type (WT): homozygous *dPDZ-GEF*$^{E-174/E-174}$: hs-FLP: *dPDZ-GEF*$^{E-174}$,FRT^{40A}/ubi-GFP,FRT^{40A}. (A,B) Fasciclin III (Fas) is expressed at the membrane of socket (brown) and hair (yellow) cells, and neurons (anti-Elav), and partly colocalizes with F-actin . (C,D) The localization of Fasciclin is unaffected by *dPDZ-GEF*$^{E-174}$. (C-F) Socket cells are abnormally elongated in mutant tissue (arrowheads) compared to wild-type tissue (GFP). All images were acquired by confocal microscopy with a 63X objective lens and zoomed 2 fold. (the neurons are labelled with anti-Elav)

Figure 7: Characterization of septate junctions in *dPDZ-GEF* mutants (II)

The state of cell adhesion between the cells of sensory organ was further examined by assessing the integrity of adherens junctions at the pupal anterior wing margin. Thus, the localization and expression of Armadillo, the *Drosophila* homolog of β-catenin, was visualized by antibody staining (Figure 8). At 28h APF, confocal miscroscopy reveals that Armadillo and filamentous actin (F-actin) colocalize at most apical region of the wing margin, and are also concentrated within puncta located at what seems to be the junction between the prospective hair root and the socket cell (A^i). The same was observed with E-cadherin (data not shown). Moreover, Armadillo appears to be faintly expressed in the

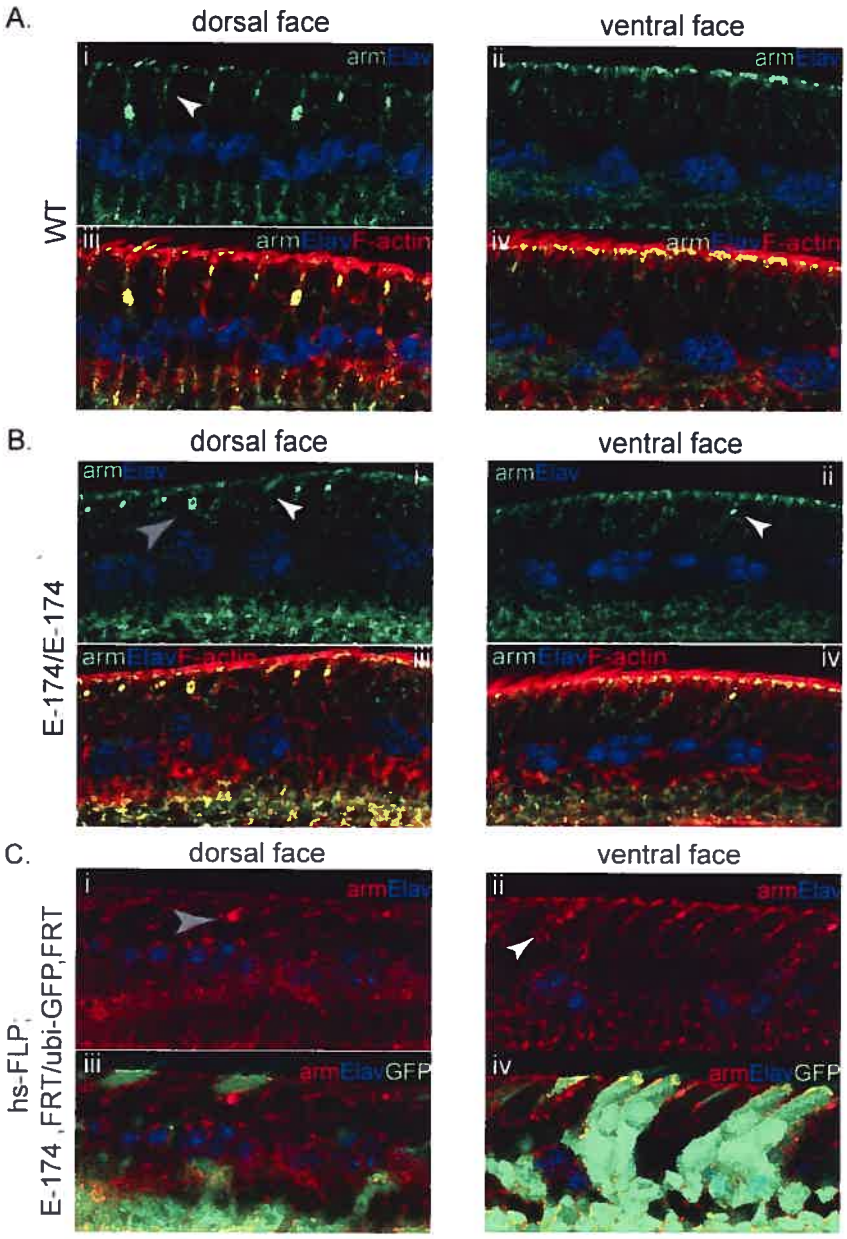

Figure 8. A *dPDZ-GEF* allele disrupts the localization of adherens junction protein Armadillo. (A-C) Dorsal and ventral face of the anterior wing margin of pupae at 28h APF for the following genotypes: wild-type (WT), homozygous *dPDZ-GEF*$^{E-174/E-174}$, *hs-FLP; dPDZ-GEFE-174,FRT*^{40A}*/ubi-GFP,FRT*^{40A}. (A and B) Armadillo colocalizes at the apical region of the anterior wing margin, and whithin puncta (arrowheads) that are unevenly distributed in respect to neurons (anti-Elav) in a given focal plane in *dPDZ-GEF* mutants. (C) The phenotype is observed in mutant *dPDZ-GEF*$^{E-174}$ clones when compared to WT clones (GFP). All images were acquired by confocal microscopy with a 63X objective lens and zoomed 2 fold. (white arrowheads correspond to mechanosensory bristles, grey arrowheads correspond to chemosensory bristles)

Figure 8: Characterization of adherens junctions in *dPDZ-GEF* mutants

cytoplasm. Cell membranes (labelled with Phalloidin-TRITC) are visualized with difficulty in individual focal planes in flies homozygous for *dPDZ-GEF*$^{E-174}$, an indication of cellular disorganization at the wing margin (compare B^{iii} with WT in A^{iii}). Consequently, the expression of Armadillo cannot be assessed within the cyplasm of sensory organ cells. However, hair root/socket puncta appear to be unevenly distributed in respect to neurons (labelled with anti-Elav) within a focal plane (compare B^{ii} and B^{iii} with WT in A^{ii} and A^{iii}), suggesting that hair and socket cells are not appropriately localized in homozygous *dPDZ-GEF*$^{E-174/E-174}$ pupae. Furthermore, we have induced mitotic clones of *dPDZ-GEF*$^{E-174}$, and mutant tissue appears to have the same defects when compared to wild-type tisuue (GFP) (C). Finally, the same phenotypes were observed in transheterozygous *dPDZ-GEF*$^{E-322/E-696}$ pupae (data not shown).

In conclusion, *dPDZ-GEF* alleles seem to disrupt the localization of Armadillo at cell membranes, and do not affect the expression oof Dlg nor Fas III. Consequently, I propose that dPDZ-GEF activity may play a role in the localization of adherens junctions within the wing margin, or may influence cell shape in such a way that adherens junctions become mislocalized.

3.1.5 dPDZ-GEF may regulate the distribution of the α and β integrin subunits in the pupal anterior wing margin

Since Rap1 is known to modulate actin dynamics through the regulation of cadherins and integrins, the expression pattern of integrins at the anterior wing margin was assessed in wild-type and *dPDZ-GEF* mutants (Figure 9). At 28h APF, both αPS1 and βPS integrin subunits exhibit a faint and punctate expression pattern at the cell membrane of sensory organ cells (Figure 9 A and C). The αPS2 subunit is not expressed at the wing margin (data not shown), thus the integrin heterodimer found at the anterior margin of pupae appears to be αPS1/βPS. Homozygous *dPDZ-GEF*$^{E-174'E-174}$ and transheterozygous *dPDZ-GEF*$^{E-322E-696}$ pupae exhibit irregular expression of βPS (B). In some cases the distribution of βPS at cell membranes appears to be absent (B^{i} and B^{ii}), in other cases βPS appears to be aberrantly accumulated at cell membranes (B^{iii}, B^{iv} and B^{v}). Since the expression of αPS1 is weak at the anterior wing margin, it is difficult to assess whether

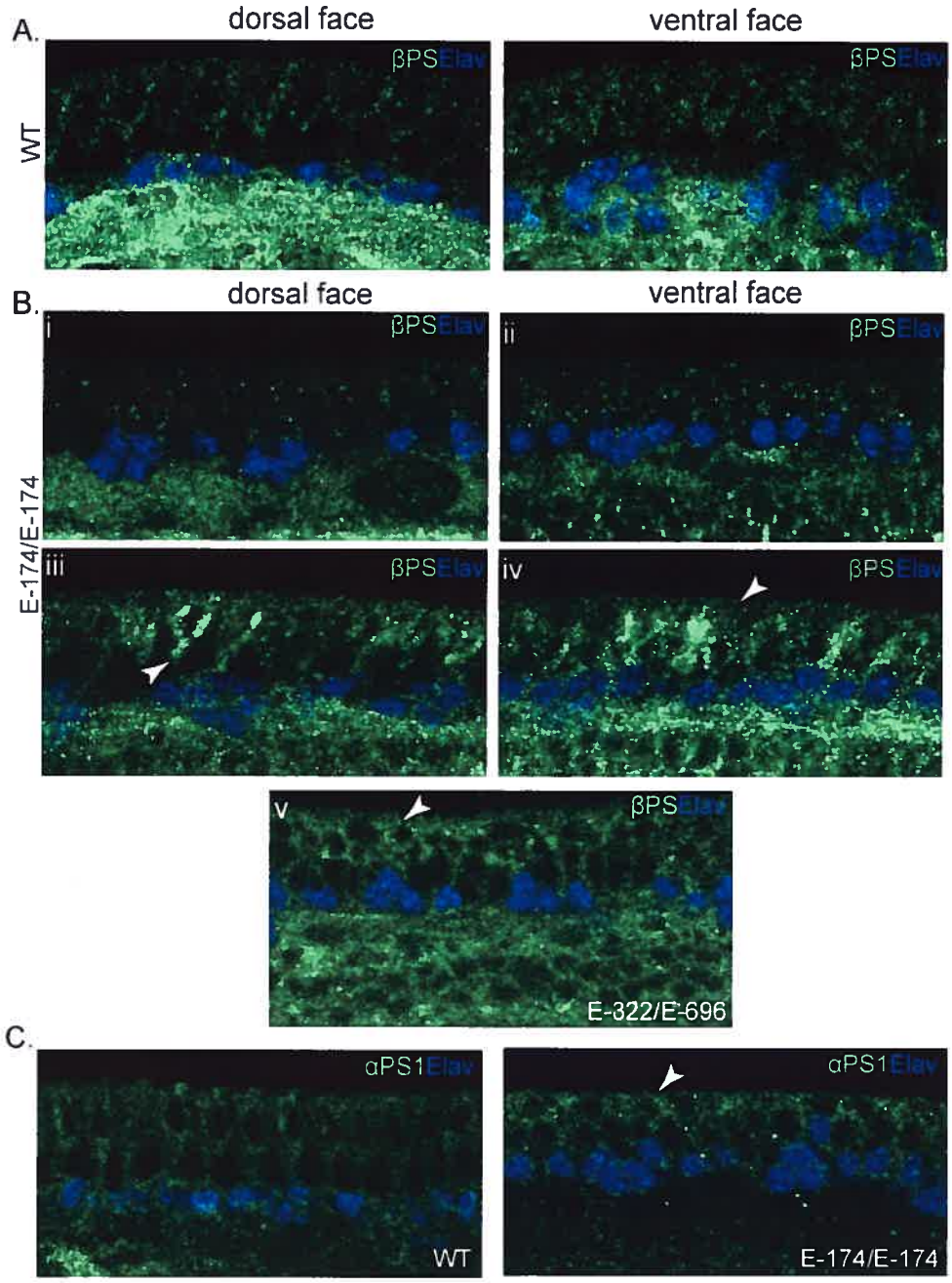

Figure 9. *dPDZ-GEF* mutant alleles disrupt the localization of αPS1 and βPS integrin subunits. (A-C) Dorsal and ventral face of the anterior wing margin of pupae at 28h APF for the following genotypes: wild-type (WT), homozygous *dPDZ-GEF*$^{E-174/E-174}$, transheterozygous *dPDZ-GEF*$^{E-322/E-696}$. (A and C) βPS and αPS1 have a punctate distribution at the membrane of sensory organ cells. (B) βPS is either lost (i and ii) or aberrantly accumulated (white arrowhead in iii, iv and v) at cell membranes in *dPDZ-GEF* mutants. (C) αPS1 is abberantly accumulated at cell membranes in *dPDZ-GEF* mutants (white arrowhead). All images were acquired by confocal microscopy with a 63X objective lens and zoomed 2 fold.

Figure 9: Characterization of integrins in *dPDZ-GEF* mutants

dPDZ-GEF alleles affect its expression pattern. Nonetheless, αPS1 seems to accumulate at cell membranes in homozygous *dPDZ-GEF*$^{E\text{-}174\dot{E}\text{-}174}$ pupae when compared to wild-type (Figure 9C). Thus, both αPS1 and βPS integrin subunits appear to be aberrantly localized at the anterior wing margin in *dPDZ-GEF*^{mutant} pupae. These results suggest that dPDZ-GEF may play a part in the distribution of the αPS1/ βPS integrin heterodimer at cell membranes during pupal wing development.

3.1.6 *dPDZ-GEF* alleles genetically interact with *cnk*^{CT}

Since the *dPDZ-GEF* alleles used to characterize dPDZ-GEF function were isolated from the CNK^{CT} screen described in section 1.5.1, we investigated whether *cnk*^{CT} could influence wing margin patterning in a way that is similar to the effects produced by *dPDZ-GEF* mutations. Gal4^{C96}, which induces the expression of the Gal4 protein as early as 80h AEL, was used to drive the expression of *cnk*^{CT} in the developing wing margin [298, 299]. The targeted overexpression of the carboxy-terminus of CNK in the wing margin induces phenotypes reminiscent of the wing scalloping induced by mutations in members of the Notch pathway (Figure 10A) [300]. For instance, nine percent of adult flies expressing *cnk*^{CT} in the wing margin (*C96> CNK*^{CT}) exhibit a "notched" anterior wing margin (AWM) phenotype (Figure 10B). Then, *cnk*^{CT} was expressed in combination with one copy of a *dPDZ-GEF* allele to assesss whether the genetic interaction between the *pdz-gef* and the *cnk* loci observed in the adult *Drosophila* eye (refer to section 1.5.1) is maintained in the anterior wing margin. Thus, the expression of one mutant *dPDZ-GEF* allele (E-322, E-174, E-696, or null P13720) causes the percentage of flies with "notched" anterior wing margins to increase to at least 17%, and up to 53% (Figure 10B).

These results suggest that *dPDZ-GEF* alleles enhance the penetrance of the "notched" AWM phenotype in *C96>CNK*^{CT} flies, confirming that PDZ-GEF and CNK interact in the anterior wing margin as they do in the eye. Thus, dPDZ-GEF and CNK might work together or in parallel to regulate wing and eye morphogenesis. However, these results should be interpreted with caution as a single *UAS-CNK*^{CT} transgenic line was used, and consequently, genetic interaction should be confirmed with other *UAS-CNK*^{CT} lines.

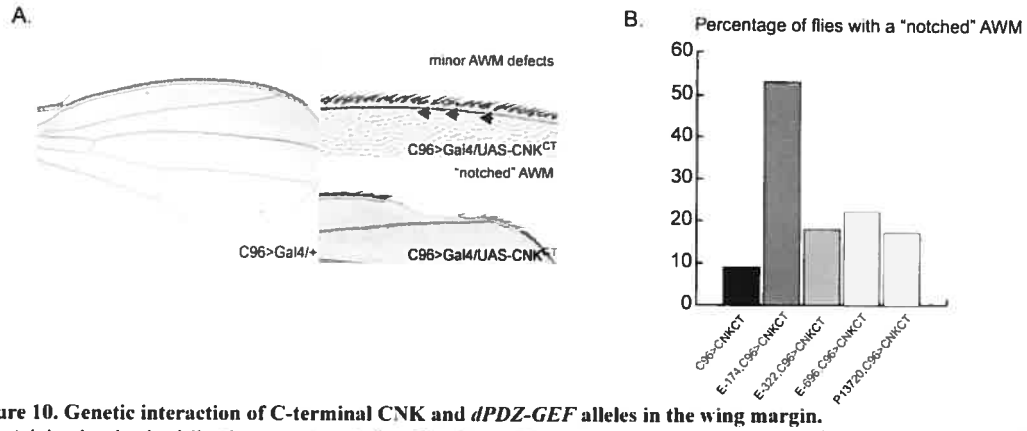

Figure 10. Genetic interaction of C-terminal CNK and *dPDZ-GEF* alleles in the wing margin.
(A) Adult wins for the following genotypes: *C96-Gal4/+* (C96>Gal4) and *C96-Gal4/UAS-CNK^CT* (C96>CNK^CT). *CNK^CT* induces anterior wing margin (AWM) defects. (B) Graph represents the percentage of flies with a notched AWM wing margin: C96>CNK^CT (9%). E-174/+;C96>CNK^CT (53%). E-322/+;C96>CNK^CT (18%). E-696/+;C96>CNK^CT (22%). E-P13720/+;C96>CNK^CT (17%).

Figure 10: *dPDZ-GEF* alleles genetically interact with cnk^{CT}

1.1 Production and characterization of an anti-dPDZ-GEF antibody

Since the characterization of *dPDZ-GEF* alleles has revealed that the GEF is involved in the spacing of sensory bristles, the cellular morphology of sensory organs and the distribution of adhesion molecules during pupal development, it would be pertinent to assess the localization of dPDZ-GEF in wild-type and mutant wing discs, as mutations in the locus may cause aberrant protein expression or localization. Consequently, an antibody against dPDZ-GEF was developed. The various steps in the generation and the characterization of the antibody are described below.

1.1.1 Generation of the antibody

A non-conserved carboxy-terminal portion of 412 amino acids was used to generate a GST-dPDZ-GEF^{CT} fusion protein (Figure 11A) which was bacterially expressed and purified on a glutathione-sepharose column. The protein extract appeared to be a combination of peptides of varying length on a polyacrylamide gel stained with coomassie blue (Figure 11B). Immunobloting against the GST protein revealed that the peptides all contained the GST tag (Figure 11B), suggesting that the protein extract is a mixture of GST-dPDZ-GEF^{CT} fusion proteins of various lengths. Thus, this protein extract was used to raise a rabbit polyclonal antibody.

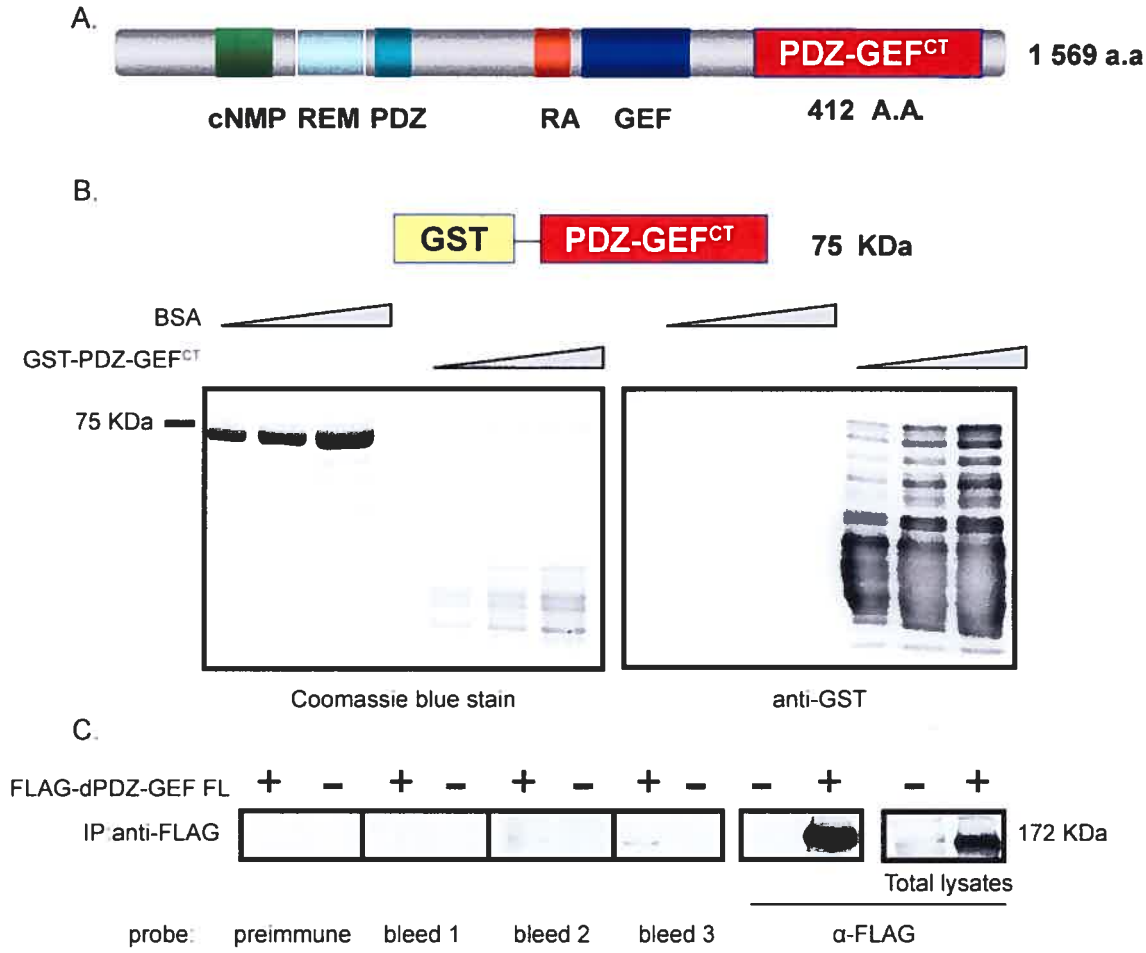

Figure 11. Generation of the anti-dPDZ-GEF antisera.
(A) The dPDZ-GEF peptide used to generate the antibody is a 412 amino acid (A.A.) non-conserved carboxy-terminal portion. (B) The GST-PDZ-GEF^{CT} fusion protein was purified from E.coli and increasing amounts (5, 10, 20 µg) were loaded on an acrylamide gel along with increasing amounts of BSA. Coomassie blue staining and immunoblotting with anti-GST reveal peptides of varying lengths. (C) The rabbit antisera were tested against transfected dPDZ-GEF: pre-immune bleed, first bleed, second bleed, and third bleed. The pre-immune and first bleeds show no immunoreactivity, while the second and third bleeds do, but not as significantly as anti-FLAG.

Figure 11: Production and characterization of an anti-dPDZ-GEF antibody (I)

1.1.2 Efficiency of the anti-dPDZ-GEF antibody

Sigma Genosys provided a pre-immune bleed, and three antisera obtained at different stages of the immune challenge. First, we tested the immunogenicity of the four bleeds against transfected and endogenous dPDZ-GEF extracted from S2 cells by

immunoblot. The pre-immune bleed and the first bleed have no reactivity against transfected dPDZ-GEF purified from S2 cells, while the second and third bleeds do (Figure 11C). Although the third bleed is the most immunoreactive antiserum, it appears to be less efficient against tagged dPDZ-GEF than the anti-FLAG antibody in western blots and does not detect endogenous dPDZ-GEF in S2 cells (Figure 11C).

In order to increase the efficiency of the anti-dPDZ-GEF antibody, the third bleed was positively purified on a column coated with MBP-dPDZ-GEF^{CT} fusion protein, and subsequently concentrated. The same treatment was used for the pre-immune bleed. The immunoreactivity of purified and purified/concentrated antisera was then tested by immunoblot against bacterially purified and transfected dPDZ-GEF protein. Since it is more effectively expressed in S2 cells, a truncated dPDZ-GEF (PDZ-GEFΔcNMP) which misses the cNMP domain was used for the purpose of this experiment. The purified and purified/concentrated anti-dPDZ-GEF antisera are immunoreactive against bacterially expressed dPDZ-GEF (Figure 12A). However, the purified/concentrated antiserum is the only one that seems to produce a signal against transfected dPDZ-GEF in total cell lysates (Figure 12A). Nonetheless, the purified/concentrated antiserum does not detect endogenous dPDZ-GEF. Moreover, the purified/concentrated antiserum is much less effective as a probe against transfected dPDZ-GEF than anti-FLAG in total S2 cell lysates. Consequently, the purification and concentration of the anti-dPDZ-GEF antibody did not significantly improve the efficiency of the antiserum against transfected and endogenous dPDZ-GEF.

Finally, the immunogenicity of the third bleed was tested by immohistochemistry in larval wing discs. The purified/concentrated antiserum appears immunoreactive compared to the pre-immune bleed (Figure 12B). However, the antibody did not label specific regions of the wing disc nor particular cellular compartments. Consequently, mitotic clones for the null allele *dPDZ-GEF*^{P13720} were generated in order to assess the specificity of the anti-dPDZ-GEF antiserum. The antibody labelled uniformly mosaic eyes and wing discs (data not shown). Thus, the anti-dPDZ-GEF antiserum does not effectively detect endogenous PDZ-GEF in *Drosophila* wing and eye disc.

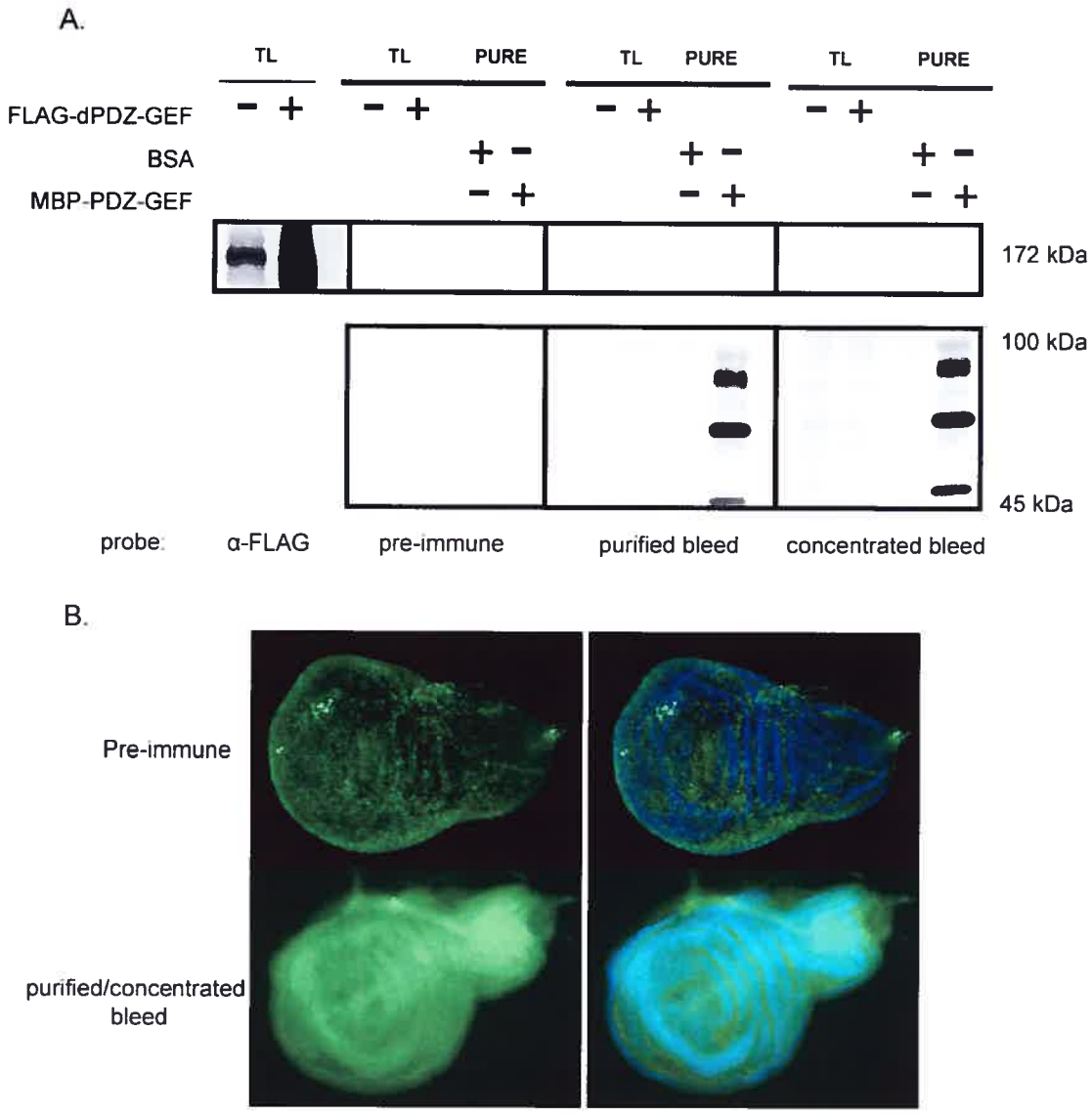

Figure 12. Efficiency of the anti-dPDZ-GEF polyclonal antibody.
(A) The efficacy of anti-Flag, pre-immune, purified and purified/concentrated bleeds was tested against transfected FLAG-dPDZ-GEFΔcNMP in total S2 cell lysates (TL) and purified MBP-dPDZ-GEF^{CT} fusion protein (PURE) by western blot (probes: purified/concentrated pre-immune bleed, purified third bleed, purified/concentrated third bleed). The anti-dPDZ-GEF antibody is less immunoreactive against transfected dPDZ-GEF than against the purified fusion protein. (B) The antibody was tested by immunohistochemistry on wild-type larval wing discs (purified/concentrated pre-immune and third bleed). anti-PDZ-GEF exhibits an unspecific pattern.

Figure 12: Production and characterization of an anti-dPDZ-GEF antibody (II)

Since the antiserum does recognize some forms of dPDZ-GEF (Figure 12A), further optimization might produce an effcient anti-dPDZ-GEF antibody that can be used for western blots or immunohistochemistry.

3.3 Expression of dsRNA against dPDZ-GEF in S2 cells

In the course of this study, mutant alleles were used as tools to study the function of dPDZ-GEF. Though there are indications that *dPDZ-GEF*$^{E\text{-}174}$, $^{E\text{-}322}$ and $^{E\text{-}696}$ are loss of function alleles (refer to section 3.1.1), their effect on *dpdz-gef* expression is not defined at this time. Consequently, the phenotype induced by a true loss of function of dPDZ-GEF should be assed in order to confirm the phenotypic characterization of *dPDZ-GEF* mutant alles described in the previous sections of this chapter.

Another group has previously reported that the null allele *dPDZ-GEF*^{P13720} is recessive lethal, and that escapers hatched into adult flies at a frequency of 1/10 000 [267]. Consequently, another approach was developed to characterize loss of function phenotypes. With the aim of generating a transgenic line expressing dPDZ-GEF dsRNA, a pWIZ construct was generated by M. Therrien to drive the expression of dsRNA against dPDZ-GEF under the control of a UAS enhancer (refer to section 2.5) [301]. The effectiveness of this construct at knocking down dPDZ-GEF expression was then assessed in S2 cells expressing FLAG-dPDZ-GEFΔcNMP. Immunoblots of total cell lysates reveal that the pWIZ construct efficiently knocks down dPDZ-GEFΔcNMP upon Gal4 expression (Figure 13). In this case, a complete knock-out of the protein is not achieved by the expression of pWIZ-dPDZ-GEF^{RNAi}, and this may probably be caused by the transient expression of the construct in S2 cells. However, a stable expression of pWIZ-dPDZ-GEF^{RNAi} in S2 cells or the fly itself may actually lead to a significant knock-down or a complete knock-out of the protein. As a result, the next step will be to generate a transgenic line expressing pWIZ-dPDZ-GEF^{RNAi}. This transgenic line could be used along with tissue- and cell-specific Gal4 drivers to knock-down the expression dPDZ-GEF in selected tissues such as the wing disc, and confirm the findings obtained with specific dPDZ-GEF mutations.

Figure 13. Expression of dsRNA against dPDZ-GEF.
The pWIZ-dPDZ-GEF^{RNAi} construct (UAS-dsRNA dPDZ-GEF) was used to produce double-stranded RNA against transfected truncated dPDZ-GEF (FLAG-dPDZ-GEF FLΔcNMP) in S2 cells, and its efficiency was assessed by western blot. When Gal4 drives the expression of increasing amounts of dsRNA, the expression of dPDZ-GEF is proportionally reduced. dPDZ-GEF was detected with anti-FLAG, and we probed Src42A as a loading control.

Figure 13: Expression of dsRNA against dPDZ-GEF in S2 cells

CHAPTER 4 : DISCUSSION AND CONCLUSION

4.1 Characterization of the function of PDZ-GEF

The main objective of my Master's project was to study the function of PDZ-GEF via the characterization of the phenotypic effects induced by *dPDZ-GEF* mutant alleles recovered from a genetic screen for genes linked to cnk. The results obtained and future perspectives are discussed in the following sections.

4.1.1 dPDZ-GEF and the regulation of wing margin patterning

The finding that *dPDZ-GEF* alleles disrupt the distribution of sensory bristles along the anterior wing margin of adult flies (Figure 3) has raised questions about the function of this Rap-GEF. Interestingly, the number or the macroscopic morphology of sensory bristles is not affected in mutants, suggesting that dPDZ-GEF is not involved in their differentiation or the specification of SOPs during larval development. As mentioned in section 1.4.1.2.2, Wingless and Notch signalling cooperate in order to pattern the wing margin early in larval development [285]. Going as early in *Drosophila* development as the third larval instar, the expression of wing margin determinants such as Wingless, and Notch signalling members Cut and Scabrous were not affected by *dPDZ-GEF* alleles (Figure 4). Thus, these results suggest that dPDZ-GEF is not involved in early wing margin patterning via the Notch or Wingless pathways.

Nonetheless, morphological wing defects were observed during pupal life as early as 16h APF (data not shown). Indeed, the shape and localization of cells within sensory organs was shown to be altered in *dPDZ-GEF* mutant pupae (Figure 5). However, there appears to be no supernumerary cells nor any missing ones, thus confirming that dPDZ-GEF is not be involved in cell fate commitment of SOPs. Intriguingly, only the mechanosensory bristles seem to exhibit spacing defects in the adult fly. Besides confirming that chemosensory bristles are innervated by five neurons, no significant differences in the morphology of mechanosensory and chemosensory bristles have been revealed, as their morphology appears to be equally disturbed by *dPDZ-GEF* alleles. Moreover, the distribution of adhesion molecules armadillo and integrin αPS1/βPS in both

classes of sensory bristles appears to be equally altered in *dPDZ-GEF* mutants (Figures 8 and 9). Consequently, *dPDZ-GEF alleles* specifically alter the spacing of mechanosensory bristles in adults and pupae (Figures 3 and 5), though the cellular morphology of both chemo- and mechanosensory is altered. Since the formation of sensory organ precursors of chemo- and mechanosensory is regulated by different proneural proteins, it is possible that dPDZ-GEF differentially regulates the spacing of SOPs on the prospective anterior wing margin [289]. However, the specificity of the spacing defect may also be due to other factors that render chemosensory bristles impervious to misalignement after SOP specification. Either way, this aspect of dPDZ-GEF function was not specifically addressed in this work, and will need to be further investigated in the future.

4.1.2 dPDZ-GEF and the regulation of cell adhesion molecules

The finding that the distribution of adhesion molecules Armadillo (*Drosophila* β-catenin) and integrins αPS1/βPS is altered at the anterior wing margin of pupae (Figures 8 and 9) suggests that dPDZ-GEF plays a role in their cellular localization.

On one hand, the puncta in which Armadillo is accumulated at the hair/socket cell junction are aberrantly localized in *dPDZ-GEF* mutants (Figure 8). This could be a consequence of the morphological defects induced by *dPDZ-GEF* alleles, or it could be that dPDZ-GEF directly regulates the localization of adherens junction proteins in the wing margin. Interestingly in the wing disc epithelium, *R* mutant mitotic clones exhibit aberrant localization of adhesion proteins, including Armadillo, within new adherens junction following mitosis [178]. In parallel, Rap1 establishes cell polarity in yeast bud-site selection and mammalian neurite extension via its activation of Rho family GTPases [123, 124, 126, 127]. However, PDZ-GEF/Rap1 signalling in *D. discoideum* appears to regulate the establishment of cell polarity by stimulating substrate adhesion [250, 265]. Thus, one can suppose that Rap1 positively regulates cell polarity via Rho GTPases, and that PDZ-GEF/Rap1 signalling regulates chemotaxis by influencing the balance between substrate adhesion and cell polarity/migration. Interestingly, SOPs in the peripheral nervous system in *Drosophila* give rise to sensory bristles via asymmetric cell division of precursor cells,

which regulates the distribution of cytoplasmic determinants between daughter cells [302]. This polarization is caused by the changes in the orientation of the mitotic spindle relative to adherens junctions as cell division of precursor cells progresses [152, 302]. Furthermore, the downregulation of adherens junction components such as E-cadherin disrupts the orientation of the mitotic spindle in precursor cells that give rise to bristle and socket cells, alters their subsequent localization in respect to the body axis [152]. However, this defect does not seem to affect the asymmetric distribution of polarity regulators Bazooka, *Drosophila* PAR-3, and Pins [152]. In parallel, I have demonstrated that the localization of adherens junction protein, Armadillo, is impaired in *dPDZ-GEF* mutants, and that the shape of bristle and socket cells is disrupted in theses mutants. Thus, dPDZ-GEF may be involved in the formation of adherens junctions during sensory organ development, and mutant *dPDZ-GEF* alleles may lead to aberrant asymmetric cell division and subsequent morphological defects in the anterior wing margin. This hypothesis could be tested by monitoring SOP cell division and the assembly of new adherens junctions early in the development of wild-type and *dPDZ-GEF* mutant pupae by time lapse microscopy.

On the other hand, the distribution of αPS1 and βPS integrin subunits is altered in *dPDZ-GEF* mutants (Figure 9). Intriguingly, *dPDZ-GEF* alleles induce the absence and the accumulation of integrins in the anterior wing margin of pupae. In *Drosophila*, loss of function mutations in integrin subunits induce phenotypes such as wing blisters in adults and incomplete dorsal closure in embryos, which are both caused by the failure to establish cell adhesion between independent cell layers during morphogenetic events [303]. Concurrently, integrins are also involved in axon pathfinding and synaptic plasticity in the embryonic central nervous system (CNS) and in larval neuromuscular junctions [304, 305]. Thus, the delamination observed in axonal fascia of *dPDZ-GEF* mutant pupae, as well as the aberrant orientation of axons and the detachment of neurons, may be caused by the abnormal distribution of integrins in the wing margin (Figure 5).

In conclusion, dPDZ-GEF seems to be involved in the regulation of adhesion via integrins and cadherins, transmembrane proteins that are linked to the actin cytoskeleton

via proteins such as talin and β-catenin respectively [137, 167]. Over the short time in which PDZ-GEF has been studied, the fruit fly has been the only model in which a functional relationship between PDZ-GEF/Rap1 signalling and E-cadherin/β-catenin- or integrin-mediated cell adhesion has been demonstrated [268, 269, 271]. Nonetheless, there is a significant amount of evidence showing that mammalian Rap1 regulates both cadherin- and integrin-mediated cell adhesion (refer to sections 1.3.2.4 and 1.3.2.5). Unfortunately, the role of Rap1 has not been investigated during the course of this study. It will be important in the future to verify whether the phenotypic effects of *dPDZ-GEF* mutant alleles are related to aberrant Rap1 signalling.

4.1.3 Insight on the biochemical function of *dPDZ-GEF*

In order to fully comprehend the phenotypes observed in *dPDZ-GEF* mutants, we must address the biochemical activity of the mutated proteins. Although the hypothesis has not been tested biochemically with GEF assays, mutations within conserved residues of the GEF domain (E-322 and E-696) are likely to partially or completely block the catalytic activity of dPDZ-GEF (Figure 2). Consequently, *R* might be downregulated in cellular compartments where dPDZ-GEF is found, thus producing phenotypic anomalies, such as aberrant spacing of wing margin bristles, as a result. Intriguingly, dPDZ-GEF alleles that harbour a mutation in the cNMP domain (E-174) or in the GEF domain induce similar phenotypes in homozygous or transheterozygous combinations (Figure 3). This suggests that a mutation within the cNMP domain can potentially have the same functional effect as mutations likely to cause a loss of GEF activity. Though no function has been attributed to the cNMP domain of PDZ-GEF proteins to date, the substitution of aspartic acid (Asp) to a valine residue found in the $dPDZ\text{-}GEF^{E\text{-}174}$ allele has highlighted a region of the cNMP domain that contributes to dPDZ-GEF activity. In Epac proteins, the cNMP domain sterically blocks the GEF domain, and this auto-inhibitory hold is released by the binding of cAMP (refer to 1.3.3.2.3). Correspondingly, that specific Asp residue of dPDZ-GEF might be involved in the control of an inhibitory mechanism that constrains the catalytic activity of the protein. If that is the case, the substitution of this residue for any other could

potentially prevent adequate control of GEF activity, and disrupt Rap1 regulation within specific cellular compartments. Conclusively, the activity of mutant dPDZ-GEF proteins remains to be tested in order to confirm that the phenotypes recovered in *dPDZ-GEF* mutants are due to a loss of function, as well as to clarify the action of the cNMP domain on catalytic activity.

4.1.4 Development of tools for the study of dPDZ-GEF

Since dPDZ-GEF appears to regulate the distribution of cell adhesion molecules, there is a possibility that dPDZ-GEF is itself aberrantly localized within the wing margin of mutants. In order to test this hypothesis, an anti-dPDZ-GEF polyclonal antibody was generated. However, when it was tested by western blot and by immunohistochemistry it proved to lack efficacy, even after its purification and concentration (Figures 11 and 12). Nonetheless, the anti-dPDZ-GEF antibody can recognize the transfected protein after purification from S2 cells (Figure 11). Moreover, it is quite effective at recognizing particular peptides in MBP-dPDZ-GEF^{CT} protein extracts (Figure 12), which are mixtures of MBP-containing peptides much like those found in GST-dPDZ-GEF^{CT} protein extracts (Figure 11 and data not shown). Thus the polyclonal antibody exhibits a certain degree of immunoreactivity against the dPDZ-GEF. Interestingly, Steven X. Hou and his group have generated another dPDZ-GEF antibody in rabbits, that has been used successfully to visualize endogenous dPDZ-GEF in *Drosophila* testes and spermathecae [268, 269]. The peptide used to generate this antibody was composed of the twenty amino acids (1548-1567) located at the carboxy-terminus of the annotated dPDZ-GEF protein sequence available on Flybase (http://flybase.bio.indiana.edu/). The dPDZ-GEF peptide used to generate our antibody was significantly longer (412 amino acids), but contained the twenty residue stretch used by the Hou group. Consequently, the GST-dPDZ-GEF^{CT} fusion protein could have generated a larger pool of antigenic sequences during the production of the antibody. It is possible that the majority of the immunoglobulins contained in our antisera target regions of dPDZ-GEF that are not accessible in the endogenous protein. Consequently, other approaches could be used to increase the efficiency of the antibody

such as denaturing proteins in immunohistochemistry preparations, or finding more appropriate incubation methods in immunoblotting and antibody staining in tissues. The problems encountered with the detection of endogenous dPDZ-GEF in *Drosophila* S2 cells may be attributed to dPDZ-GEF expression that is either weak or absent in this particular cell line. This matter could be easily addressed by performing RT-PCR on S2 cell extracts. Furthermore, a transgenic line expressing a tagged dPDZ-GEF could be generated in order to visualize the protein more effectively in *Drosophila* organs. Another antibody could also be obtained either via the generation of another peptide or the acquisition of the Hou antibody. Either way, the use of a PDZ-GEF antibody will be essential in determining the localization and the expression of the endogenous protein. Moreover, such an antibody would be helpful to study the function of dPDZ-GEF via biochemical approaches.

Finally, the phenotypic characterization of *dPDZ-GEF* mutants was performed using alleles that contain point mutations (Figure 2), and for which the effects on protein expression are not yet known. Therefore, it is essential to evaluate the phenotypic effects of dPDZ-GEF loss of function in order to validate the results obtained in the course of this study. In recent years, RNAi has proven to be effective in silencing gene expression and studying subsequent loss-of-function phenotypes in *Drosophila*. However, mere injection of dsRNA produces a transient knock-down that is not inherited [306]. A transgene containing an inverted repeat configuration of a specific gene sequence can be effectively used in *Drosophila* to produce dsRNA against a chosen gene product [307, 308]. The generation of a pWIZ-dPDZ-GEF^{RNAi} construct is the first step in producing a transgenic line that will be used to study PDZ-GEF in *Drosophila*. Moreover, an inducible expression system to perform RNAi permits the control of the time and location of the dPDZ-GEF knock-down, thus allowing the characterization of precise phenotypic effects. In the future, the pWIZ-dPDZ-GEF^{RNAi} line could also be used to investigate the functional relationship between *dPDZ-GEF* and other genes via phenotype rescue experiments and genetic screens.

4.1.5 The *Drosophila* wing margin as a model of study

In order to characterize the wing margin phenotype engendered by *dPDZ-GEF* mutant alleles, a thorough study of the development of sensory bristles was needed. Though the morphology of the adult anterior wing margin has been described, there are very few reports on the cellular morphology of the anterior wing margin. Immunohistochemistry on the pupal wing disc has facilitated the visualization of cells within sensory bristles, as well as the expression pattern of cell adhesion proteins within these cells. Images obtained by confocal microscopy were instrumental in acquiring a better understanding of the morphology of the wing margin during pupal development in order to define the phenotypes induced by dPDZ-GEF alleles. Since no current models of the pupal wing margin of *Drosophila* are available to date, I have depicted my own interpretation which is based on the data that was compiled during the course of my research (Figure 10). Though it might be the first of many models, this representation will hopefully serve as a guide for others who chose to use the *Drosophila* wing margin as a system in which to conduct their research.

In the study of PDZ-GEF function in Drosophila, the use of the wing margin phenotype as a model is attractive as it has not yet been characterized in detail. The alleles recovered from the screen and used in this study each carry a mutation that can potentially reduce GEF activity (refer to 4.1.3). In order to produce aberrant spacing of wing margin bristles, two copies of mutant dPDZ-GEF alleles are needed (Figure 3). It is would be interesting to determine whether the combination of one of these alleles and an allele carrying a mutation engineered within one of the other conserved domains of dPDZ-GEF can reproduce the wing margin phenotype in adult and developing flies. For example, if a mutation within the REM, a domain which also regulates catalytic activity, reproduced the wing margin phenotype, this would help confirm that the loss of catalytic activity in dPDZ-GEF is responsible for the phenotype. Moreover, investigating whether mutations created within the RA and the PDZ domains of dPDZ-GEF can reproduce the wing margin

phenotype might provide new insight on the importance of protein localization in the function of PDZ-GEF proteins in vivo.

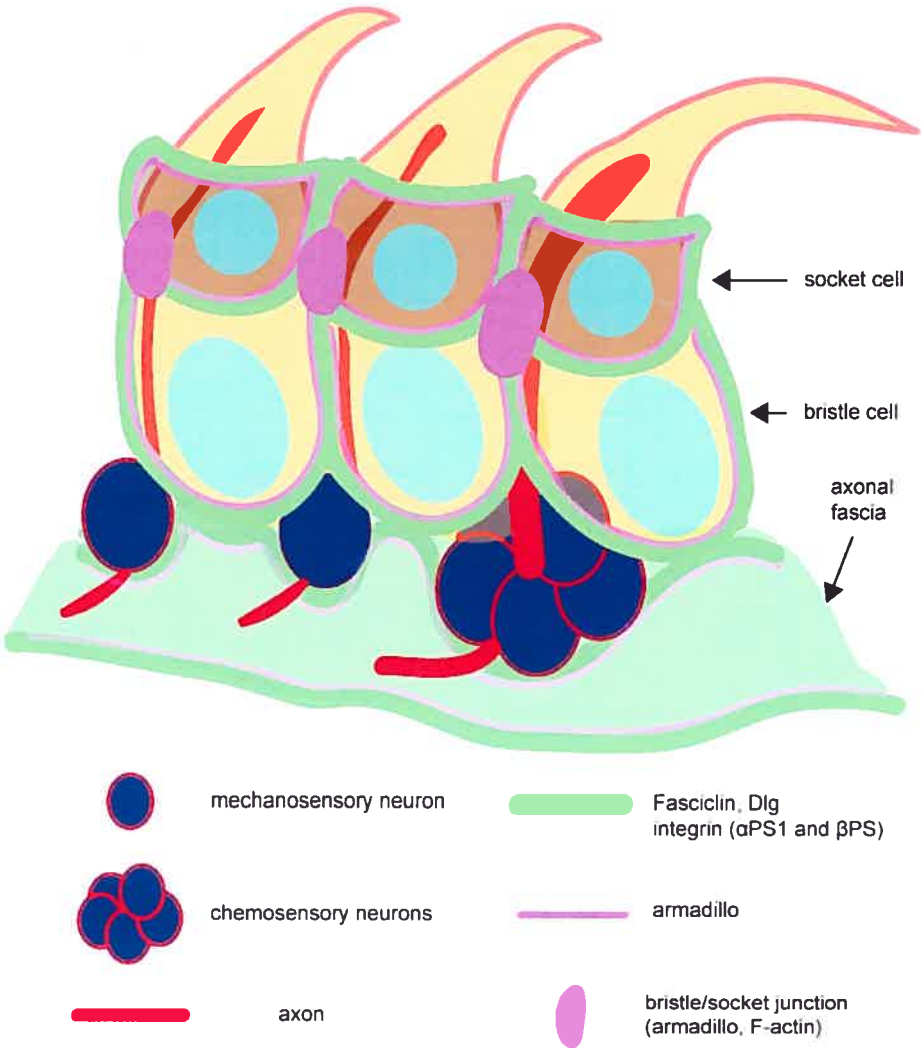

Figure 14. The pupal anterior wing margin.
This representation is based on images taken by confocal microscopy at 28hAPF. Stout mechanosensory bristles (one neuron) and chemosensory bristles (five neurons) are represented. Three sensory organ cell-types are represented: neurons (blue), bristle cells (beige) and socket cells (brown). The brisles are innervated by bipolar neurons which extend their axons to the bristle cell and the axonal fascia. Septate junction proteins Fasciclin and Dlg, as well as integrin subunits αPS1 and βPS are localized abundantly at cell membranes and in the axonal fascia, while armadillo is less abundant. However, armadillo and filamentous actin are accumulated at the junction between bristle and hair cells.

Figure 14: The *Drosophila* wing margin as a model of study

4.2 CNK and PDZ-GEF/Rap1 signalling in the regulation of actin dynamics

A functional relationship between CNK and dPDZ-GEFwas defined in *Drosophila*, as *dPDZ-GEF* alleles enhance phenotypes induced by the expression of the carboxy-terminus of CNK in the eye and anterior wing margin of *Drosophila* (Figures 2 and 10). Moreover, the CNK^{CT} genetic screen isolated alleles of *R* and *dPDZ-GEF*, but not of other Rap regulators of effectors. Thus, the potential relationship between Rap1 signalling and CNK may be exclusively credited to dPDZ-GEF activity. However, genetic interaction studies with other components of the Rap pathway have yet to be conducted in order to confirm or refute this hypothesis. Interestingly, the study described in this thesis as well as data from other studies suggest that dPDZ-GEF/Rap1 signalling and CNK may be involved in the regulation of actin dynamics from junctional complexes.

Firstly, MAGUIN-1, a human CNK2 homolog which contains a carboxy-terminal PDZ-binding motif, interacts with the PDZ domains of post-synaptic membrane scaffold proteins of PSD-95 and densin-180, which are the mammalian homologs of *Drosophila* septate junction proteins Dlg and Scribble respectively [53, 54]. Moreover, the carboxy-terminus of CNK also interacts with another septate junction component, DAL-1, which is the homolog for *Drosophila* coracle [44]. M. Therrien's group has previously reported that the carboxy-terminus of CNK has a dominant-negative effect on the Ras-MAPK pathway (refer to section 1.2.3.2). It is therefore possible that the carboxy-terminus has a dominant-negative effect on the localization of PDZ domain-interacting proteins at the cell membrane. Indeed, when expressed, cnk^{CT} might sequester other membrane scaffold proteins and prevent them from interacting with proteins that regulate cellular functions such as cell adhesion. As a result, epithelial integrity could be compromised, which would explain partly the morphological defects were observed in eye and wing discs when cnk^{CT} is expressed (Figure 2). Furthermore, the increased penetrance of the "notched" anterior wing margin phenotype upon the expression of cnk^{CT} in a *dPDZ-GEF* mutant background

is proof of a genetic interaction between dPDZ-GEF and CNK (Figure 10). Interestingly, both CNK and PDZ-GEF harbour PDZ domains, and mammalian isoforms are known to hold PDZ-interacting motifs as well. However, no direct interaction between PDZ-GEF and CNK has been reported to date.

Secondly, mammalian PDZ-GEF proteins are reportedly enriched at the plasma membrane, and this recruitment seems to be mediated by the PDZ domain [245, 251, 257]. Interestingly, PDZ-GEF1 was shown to interact with proteins that interact directly or indirectly with actin such as β-catenin and PDZ domain-containing MAGUK family scaffold proteins MAGI-1 and brain-specific MAGI-2/S-SCAM, suggesting that PDZ-GEF is targeted to the plasma membrane via protein-protein interactions involving PDZ domains [257-259]. MAGI-1 was also recently shown to be required for Rap1-dependent enhancement of cell-cell adhesion via VE-cadherin in the vascular epithelium [187]. Consequently, membrane scaffold proteins may recruit CNK and PDZ-GEF to junctional complexes in order to promote signal transduction regulating cell adhesion, as well as other cellular functions.

Thirdly, Knox and Brown have demonstrated that Rap1 regulates the distribution of adherens junctions in the wing disc of *Drosophila*, and exerted no regulation of septate junctions [178]. *dPDZ-GEF* alleles induce delamination within the axonal fascia of the pupal anterior wing margin (Figure 5). In the pupal wing disc, glial cells that originate from SOP differentiation ensheath the axonal fascia by adhering directly to axons, and migrating along the developing fascia [309]. Interestingly, septate junction proteins Neurexin IV, Contactin and Neuroglian, are involved in axonal ensheathment in *Drosophila* peripheral nervous system [297]. However dPDZ-GEF alleles recovered from the CNK^{CT} screen do not seem to affect the distribution of septate junction proteins Dlg and Fasciclin (Figures 6 and 7). Consequently, the role of dPDZ-GEF in axonal ensheathment should be investigated in further details, starting with the assessment of Neurexin IV, Contactin and Neuroglian expression and localization in the anterior wing margin of wild-type and mutant

pupae. Since CNK appears to be located within septate junctions, the phenotypic effects of cnk^{CT} should also be assessed as defects in axonal ensheathment may also occur in mutants.

Finally, PSD-95, a MAGUK family protein not only recruits CNK to junctional complexes, it recruits Rap-GAPs of the SPA-1 family as well[213, 214, 310]. Thus, CNK, PDZ-GEF, and SPA-1 family Rap-GAPs are all recruited within protein complexes via PDZ domain-mediated interactions at the cell membrane of mammalian post-synaptic junctions, and possibly at sites of cell adhesion in epithelial tissues and axonal structures in *Drosophila*. The localization of Rap1 regulators within junctional complexes promotes the recruitment of the small GTPase to the membrane and controls its influence on actin dynamics. Furthermore, reports that CNK interacts with members of Rho GTPase signalling suggest that CNK may also influence cytoskeletal rearrangements from junctional complexes [47, 49, 50, 52]. Hence, it is possible that epithelial integrity is partly maintained by Rap1 signalling via Rap1 regulators located at cellular junctions such as SPA-1 GAPs, PDZ-GEF and potentially CNK.

4.2.1 Signal transduction networks

The lingering question in the study of PDZ-GEF concerns its activation. Other Rap-GEFs such as Epac, C3G and CalDAG-Gef are activated by specific molecular signals. However, no mode of activation has been described for PDZ-GEF as of yet. In addition to the controversy concerning the nucleotide-binding capacity of the cNMP domain, it has been proposed that PDZ-GEF may be activated by Rap1-GTP thus creating a positive feedback loop [248, 249]. Furthermore, the effectors targeted by PDZ-GEF/Rap1 signalling have not yet been identified. Consequently, Dr. Therrien's laboratory is currently preparing a modifier screen based on phenotypes induced by $dPDZ\text{-}GEF^{E\text{-}174}$ expression, in order to isolate potential regulators and effectors of PDZ-GEF.

One of the reasons behind the cnk-dependent screen was to identify novel components that are involved with the Ras-MAPK pathway. The small GTPase Rap1 has a long standing history with Ras signalling, which has created a lot of controversy in the field

as stated in the introduction (refer to section 1.3.1.5). The isolation of alleles of *R* and *dPDZ-GEF* from cnk-dependent screen demonstrates yet another interaction between Rap1 signalling and the Ras-MAPK. For instance, *dPDZ-GEF* and *R* alleles induce the loss of photoreceptor cells, a sign of the disruption of the Ras-MAPK pathway (FIG). Though the significance of the interaction between the Ras-MAPK pathway and Rap1 has not been addressed in this research project, it would be relevant to address it in the future by various experimental approaches.

As mentioned above, a quick review of the literature suggests that CNK and PDZ-GEF are likely found within similar junctional complexes that involve PDZ domain-containing scaffold proteins. However, there is no evidence of an interaction between the PDZ domains and/or PDZ-binding motifs of PDZ-GEF proteins and CNK to date. Moreover, CNK and PDZ-GEF appear to be physically localized in different junctional complexes: CNK in septate junctions and PDZ-GEF in adherens junctions. Interestingly, the cnk-dependent screen conducted by our laboratory isolated predominantly alleles from the Ras-MAPK, but also provides the first indication that CNK may act as a scaffold protein in Rap1 signalling as well. There is mounting evidence that CNK may be involved in signal transduction that involves Ral and Rho GTPases (refer to 1.2.3.3). Thus, CNK may act as a signal transduction platform, which integrates intracellular signals at the plasma membrane or within junctional complexes, and directs them towards the appropriate pathway.

Conclusion

The primary goal of this research project was to characterize the function of PDZ-GEF using *D. melanogaster* as a model of study. The anterior wing margin of *Drosophila* was used as a model to study the phenotypic effects of *dPDZ-GEF* alleles on the macroscopic and microscopic morphology of the anterior wing margin. As a result, dPDZ-GEF was shown to be involved in the regulation of adherens junction proteins and of integrin subunits. Moreover, *cnk* and *dPDZ-GEF* were shown to genetically interact during

wing development, and are thought to be involved in the regulation of cell adhesion from junctional complexes. Finally, the combined action of Rap1 signalling and CNK may promote epithelial integrity via the regulation of cell adhesion. As cancer is in part characterized by the deregulation of cell adhesion, further investigation of the role of CNK and dPDZ-GEF/Rap1 signalling within junctional complexes will provide valuable insight on the regulation of cell adhesion.

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